TECHNISCHE UNIVERSITÄT WIEN
Vienna | Austria

DISSERTATION

A quantitative, time-resolved approach to bioprocess understanding for the economic production of polyhydroxyalkanoate

ausgeführt zum Zwecke der erlangung des akademischen Grades eines Doktors der technischen Wissenschaften unter der Leitung von

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Thank you note

Many friends and colleagues have accompanied and supported me on the fulfillment of this work and I would like to thank all the ones who are not listed here by name!

First and foremost I would like to thank my family, my parents, my siblings, Shaghayegh and Kami and my husband, Reza who have supported me in each and every step of the way. Thank you, Mom and Dad, for your endless love and support. Thank you Shaghayegh and Sasan for surprising me with delicious food and many road trips to the institute over the weekends. Thank you, Kami, for making me laugh every time I was feeling down. Thank you, Reza, for many long discussions on technical and scientific work and for proof-reading all my manuscripts and re-doing all the plots. Thank you, My Love, this thesis is dedicated to you for all your support, for standing by my side and loving me. You are my inspiration and the reason I have been able to complete this work and no words can express the extent of my gratitude. I just want to say thank you!

I would like to thank Professor Herwig for giving me the opportunity to work in his research group as a foreign student. For trusting in me as a Biotechnologist knowing that I had no knowledge of bioprocess engineering and for giving me a great opportunity and the responsibility to start projects from scratch with no prior background and I hope you have not regretted it. Thank you for your valuable inputs in various Jour fixes and your commitment to this working group.

Many colleagues have come and gone during my time, Alexandra Hofer introduced me to the lab as a freshman and taught me many valuable things which helped me during my research. I would like to thank Julian Kager, Paul Kroll, and Christoph Slouka for their valuable input and support.

I would also like to thank the intern and master students who have supported my work and the project: Florian Fuhrmann, Tamas Kovac, and Margit Krampl. Thank you for your engagement and good work.

Finally, I would like to take this opportunity to thank Dr. Irina Druzhinina for her support and priceless scientific contribution to this work!

Zusammenfassung

Polyhydroxyalkanoates (PHAs) kommen als Speicherstoffe natürlich in einer Vielzahl von Mikroorganismen vor. Hierbei ist Poly-3-hydroxybutyrat (PHB), das am besten untersuchteste und charakterisierteste dieser Gruppe. Es wird bei Stressbedingungen von Bakterien als Kohlenstoffreserve in Form von unlöslichen Aggregaten (sog. Inclusion Bodies) angereichert.

PHB Produktion konkurriert momentan mit landwirtschaftlicher Nutzung und wird daher als unrentable und nicht nachhaltig eingestuft. Produktion von PHB in Cyanobakterien hätte den Vorteil der der Entkopplung vom Agrarbereich, als auch die relativ billige Produktion miottels CO_2 als Kohlenstoffquelle und Licht zur Fixierung des CO_2. Jedoch hat die Produktion in Cyanobakterien zwei große Hürden: sowohl die Zellteilungsrate, als auch die spezifische Produktivität is sehr gering. Um den Schritt Richtung industrieller Verwertbarkeit zu gehen, müsste die spezifische Produktivität der Zellen drastisch gesteigert werden. Das kann zu Beispiel über genetische Modifikation („genetic engineering“) passieren, was zu rekombinanten Organismen führt. Dieses Vorgehen zur steigerung der Produktivität oder der photosynthetischen Aktivität war in mehreren Studien nicht vom erwarteten Erfolg gekrönt Mit der Kommerzialisierung und der industriellen Produktion des Produkts im Hintergrund, speziell mit Hinblick auf EU-Verodnungen basierend auf Vermeidung von petrochemisch produzierten Kundstoffen, eine Gesamtauslegung des Prozesses ist entscheidend für den Erfolg von PHB Produktion in Cyanobakterien. Geziehlte zufällige Mutation von Cyanobakterien zur Optimierung des verwendeten Stammes in Kombination mit zeitabhängiger Analyse deer Medienlimitzationen während der Produktion sind der Schlüssel für eine Steigerung der Produktion. Dieser methodische Zugang macht das Produkt markfähig und kann in Zukunft petrochemische Kunstoffe in vielen Bereichen ersetzen.

Das Ziel dieser Doktorarbeit ist es Stellschrauben für die Produktion von PHB in Cyanobakterien zu finden und diese für die Verbesserung der photsynthetisch getriebenen PHB Produktion einzusetzen. Der zeitabhängige Analyse des Prozesses umfasste drei Hauptziele:

i) Charakterisierung des Prozesses unter einem quantitaiv analytischem Licht um die Reproduzierbarkeit zu gewährleisten;

ii) die Produktioneines nicht rekombinanten industreiellen Stamms mit verbesserten Produktionsmerkmalen,

iii) Verständnis der Zellantwort auf Medienbestandteillimitationen und deren Einfluss auf die PHB Produktion um den Prozess weiter zu optimieren.

Dieses Arbeit ist in folgende Teile gegliedert:

- Ein Review über die momentanen Herausforderungen der Bioverfahrenstehnik mit Cyanobakterien.

- Das Potential der Verbesserung des photosynthetisch aktiven Stamms *Synechocystis* sp. PCC 6714 und seine PHB Produktion.
- Zeitabhängige Analyse des Prozesses vereinfacht Die prozessoptimierung und die –kontrolle. Diese analytische Methodik half um die PHB Produktion in *Synechocystis* sp. PCC 6714 erheblich zu erhöhen.
- Zufällige Mutation hat ein hohes Potential um industriell relevante Stämme für die PHB Produktion herzustellen. Witters hilft Charakterisierung der mutierten Gene mittels Sequenzierung und qPCR für die Entwicklung. Der daraus resultierende Mutant (über UV-Mutageses), MT_a24 zeigte hohes Potential und verbesserte Eigenschaften.
- Phyiologische Reaktionen auf Medienbestandteillimitationen ist essentiell um den Prozess zu optimieren und auf industrielle Massstäbe zu vergrößern. Hier konnte ein leicht skalierbarer Prozess in einem einzelnen Reaktor etabliert warden (state of the art sind mind zwei Reaktoren).
- Ein Gesamtprozess mit hohem ökonischen Potential konnte, basierend auf all diesen Schritten, in dieser Arbeit verwirklicht werden.

Die Resultate dieser Arbeit sind nicht nur für die Produktion von PHB in Cyanobakterien von höchstem Interesse, sondern können auch auf andere PHB prouzierende Organismen, wie Mikroalgen ausgeweitet werden. Diese würden, neben den Cyanobakterien, die Möglichkeit von großtechnischen benutzen Freilandanalgen bieten.

Abstract

Polyhydroxyalkanoates (PHAs) are a group of naturally occurring polymers produced by microorganisms, among which poly (3-hydroxybutyrate) (PHB) is the most studied biodegradable polymer that accumulates in bacteria as carbon reserve in the form of inclusion bodies when cells grow under stress conditions.

Current industrial PHB production processes rely mostly on the availability of agricultural resources, which are costly and unsustainable having sometimes an ecological footprint. Besides sustainability, the PHB production in cyanobacteria using CO_2 and sunlight has the advantage of reducing the production cost of this biodegradable polymer. Nevertheless, the PHB production in cyanobacteria from an economic point of view has two major drawbacks: slow cellular growth and little productivity. In order to promote photosynthetic PHB production on an industrial scale, the productivity needs significant improvement and industrially relevant cyanobacterial strains need to be optimized. Recent research has mainly focused on genetic engineering to increase PHB productivity, which mainly reports as a higher percentage of dry cell weight content. The studies have rarely reported an increase in photosynthetic efficiency or an increase in the specific growth rates or production rates.

With respect to commercialization and scale-up of the cyanobacterial PHB production, especially with the regard to EU-legislations on the petrochemical plastics, a 'holistic' approach, considering the view of the whole process, is required. A proper, quantitative time-resolved analysis of the PHB production mechanism and the physiological adaptations to media limitations may help to increase photosynthetic PHB productivity. On the basis of the mentioned hypothesis, the aim of the work described in this thesis was to explore the PHB production mechanism in cyanobacteria, using the knowledge, to improve the photosynthetic polymer production processes for industrial applications. This is needed to make cyanobacterial polymer production economically feasible and competitive with synthetic polymers and other biodegradable plastics in the market.

Within this thesis, a systematic, quantitative, time-resolved analysis of the process to prove economic feasibility of photosynthetic polymer production is presented, which is divided into three main tasks: i) Characterization of the process using a quantitative, analytical approach to guarantee a reproducible bioprocess ii) The development of industrially relevant strains with superior characteristics and iii) Understanding the metabolic responses to nutrient limitation and the influence on PHB formation to optimize the production process.

The thesis shows:

- A review of the state of the art and challenges associated with cyanobacterial bioprocess engineering.

- Unexplored potential of wild-type cyanobacteria to increase phototrophic PHB productivity, as an example the unicellular strain *Synechocystis* sp. PCC 6714 is presented as photosynthetic PHB producer.
- Quantitative, time-resolved characterization of the process facilitates bioprocess optimization and control. The approach significantly increased the polymer productivity in *Synechocystis* sp. PCC 6714.
- Random mutagenesis shows huge potential for the development of industrially relevant strains for PHB production and can help identify target genes for future genetic engineering. The selected high PHB yielding mutant, MT_a24 was generated by UV-mutagenesis showing exceptional fitness.
- Understanding the physiological responses to media limitation is essential to optimize and scale production. An easily scalable, one-step process could be established with increased PHB productivity in preference to the commonly done two-step processes.
- A bioprocess with higher productivity and economic feasibility could be developed within this thesis.

The thesis contributes towards a better understanding and set-up of an adequate process for phototrophic PHB production. The results of this work can be used to develop strategies to enhance productivity in other microalgae and cyanobacterial strains and to generate strains suitable for large-scale, outdoor cultivations.

Contents

Part I

Introduction

1) Introduction

Global plastic production reached a value of 322 million tons in 2015 (Plastics Europe, 2016). The accumulation of these non-biodegradable polymers in the environment has turned into a worldwide concern, for instance, the microplastics in the marine environment. In this context, the biodegradable polymers, due to their eco-friendly nature, offer one of the best solutions to the environmental problems caused by synthetic polymers. Biodegradable plastics are a class of bioplastics which are produced from biomass/biobased material and are commonly degraded by microorganisms and converted into simple, elemental substances such as water and carbon dioxide (Gross & Kalra, 2002).

Polyhydroxyalkanoates (PHAs) are a class of naturally occurring polymers produced in microorganisms (de Koning, 1993; Ten et al., 2015), among which poly (3-hydroxybutyrate) (PHB) is the most studied and widespread biodegradable polymer which accumulates in bacteria and some archaea in the form of inclusion bodies as carbon reserves when cells grow under stress conditions (Galia, 2010; Getachew & Woldesenbet, 2016). PHB with a high crystallinity represents properties similar to synthetic polyesters and also to polyolefins such as polypropylene (Barham & Organ, 1994; Harding et al., 2007; Madison & Huisman, 1999). In addition, due to biocompatibility and biodegradability, PHB possesses extensive interesting functions and can replace fossil-based plastics in many applications (Ten et al., 2015). However, the low elongation and break and the brittleness of PHB are limitations that can be overcome using other PHA, like blends of copolymers such as polyhydroxyvalerate (PHV) and poly (3-hydroxybutric acid-co-3-hydroxyvaleric acid) (PHBV). The copolymer can either be directly biosynthesized under varying cultivation conditions or be chemically produced in vitro. Apart from short-chain length PHA, there are medium- and long-chain-length polymers which can help to tailor the material properties (Lackner et al., 2018). Life cycle assessment studies have suggested that using PHB as a replacement of conventional petrochemical polymers lowers environmental impacts (Pietrini et al., 2007).

Today, PHB is commercially produced by heterotrophic bacteria, such as *Cupriavidus necator (C. necator)*, and recombinant *Escherichia coli (E. coli)* (Grothe & Chisti, 2000; Madison & Huisman, 1999; Schubert et al., 1988). High production cost, when compared with petroleum-based polymers, is one major challenge for extensive production and commercialization of PHB (Getachew & Woldesenbet, 2016). Major contributors to the overall cost being the expensive substrates, continuous oxygen supply, equipment depreciation, high energy demand, and

chemicals used for downstream processing (Koller et al., 2012; Nonato et al., 2001; Steinbüchel, 2008). Attention has been focused to reduce the production cost, mostly by selecting more economically feasible and efficient carbon substrates for PHB production such as whey, hemicellulose, sugar cane, agricultural wastes, and molasses (Alias & Tan, 2005; Getachew & Woldesenbet, 2016; Gurieff & Lant, 2007; Reis et al., 2003). With this background, PHB production using cyanobacteria from more sustainable resources, such as CO_2, has gained importance. Cyanobacteria are an ideal platform for the production of biofuels and bulk chemicals through

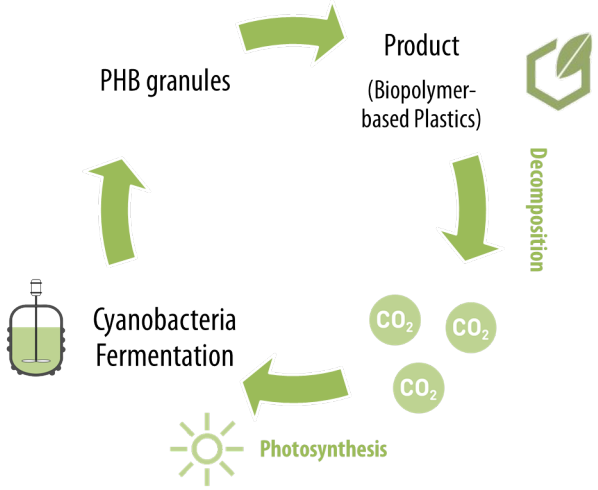

Figure 1.1: Represents the sustainable PHB production chain in cyanobacteria using CO_2 and sunlight

efficient and natural CO_2 fixation (Oliver et al., 2016). Minimization of the PHA production cost can only be achieved by considering the design and a complete analysis of the whole process (Choi & Lee, 1999). The introduction will discuss the main aspects and challenges associated with industrial production of cyanobacterial PHB. With a special focus on proper-time resolved quantification of the cyanobacterial processes, challenges associated with cyanobacterial strain engineering, the state of the art to deal with these challenges and the interlink of these topics with the goal of the thesis.

The introduction is divided into six sub-sections, namely

1.1: Commercial status of PHB
1.2: Cyanobacteria as a cell factory for PHB production
1.3: General overview of the cyanobacterial bioprocess engineering for PHB production
1.4: Challenges associated with cyanobacterial bioprocessing
1.5: Kinetics and physiology of cyanobacterial PHB synthesis
1.6: Strain improvement in cyanobacteria

1.1 Commercial status of PHB

Currently, bioplastics represent about 1 % of the total of 335 million tons of plastic produced annually (European-bioplastics, 2018). According to the latest market analysis performed by European Bioplastics, the global bioplastics production capacity is set to increase from 2.11 million tons in 2018 to approximately 2.69 million tons in 2023. The main drivers of the growth in the field of biobased, biodegradable plastics are innovative biopolymers such as polylactic acid (PLA) and PHAs. PHB production, in particular, is expected to increase to up to 100 kilotons in 2019 from approximately 34 kilotons in 2014 (European-bioplastics, 2018). The property that distinguishes PHB from petroleum-based plastics is their biodegradability. Under aerobic growth conditions, the biodegradation of PHB results in the production of CO_2 and water while under anaerobic conditions to CO_2 and CH4 (Balaji et al., 2013). PHB is compostable over a wide range of temperatures, even at a maximum of 60 °C and 55 % humidity level, 85 % of degradation of PHA has occurred within seven weeks (Balaji et al., 2013). PHA degradation in the aquatic environment is reported to occur within 254 days after disposal (Lee, 1996).

Various production plants have been established across the globe for commercial production of PHAs. The USA, China, Singapore, Germany, and Italy are the main producers of PHA using heterotrophic organisms. Europe and the Asia Pacific are expected to emerge as the largest PHA market in the near future. The European Union's competitiveness strategy, Europe-2020 which is intended for reducing greenhouse emission and promoting bioplastics industry is expected to have a positive impact on the PHA market. However, the growing sugar demands in the food and beverage industry is expected to restrict its availability for PHA production, negatively impacting the market over the next six years. Therefore it's of great importance to advance the PHA production using waste material and sustainable resources such as CO_2.

1.2 Cyanobacteria as a cell factory for PHB production

Cyanobacteria are gram-negative bacteria with a long evolutionary history and are the only prokaryotes capable of plant-like oxygenic photosynthesis (Lau et al., 2015). Unlike heterotrophic organisms, cyanobacteria require only greenhouse gas CO_2 and sunlight, along with minimal nutrients for growth, eliminating the cost of carbon source and complex media components (Lau et al., 2015). Cyanobacteria are equipped with superior photosynthetic machinery, showing higher biomass production rates compared to plants and can convert up to 3–9 % of the solar energy into biomass (Dismukes et al., 2008; Lau et al., 2015). Moreover, in contrast to plants, cultivation of cyanobacteria requires less land area, therefore cyanobacteria do not compete for arable land used for agriculture (Case & Atsumi, 2016). Some cyanobacteria can produce PHB, when essential nutrients for growth, such as nitrogen and phosphorus,

are limiting. From an economic point of view, however, photosynthetic PHB production in cyanobacteria has two major disadvantages: little productivity and slow growth (Drosg et al., 2015). Therefore, in order to promote photosynthetic PHB production on an industrially relevant scale, the productivity needs to improve significantly. Productivity is defined as the amount of PHA produced by the unit volume in unit time (Choi & Lee, 1999). In spite of all the efforts done, so far, very few studies have shown an actual improvement in the cyanobacterial PHB production process, while there are various challenges associated with cultivation, engineering and large-scale production of autotrophic cyanobacterial biomass.

1.3 General overview of the cyanobacterial bioprocess engineering for PHB production

A process lifecycle starts with process understanding and development and includes scale up and continuous process optimization until product purification. Sound process characterization is necessary for all parts of the lifecycle and in order to obtain an optimal scalable bioprocess and in-depth understanding of the bioprocess kinetics, the following points have to be known. First, the holistic knowledge of enzymatic and metabolic pathways of the PHB biosynthesis in cyanobacteria. Second, the selection or development of the optimal strain with maximum productivity. Third, the selection of inexpensive substrate and optimization of media components for the particular production strain. Fourth, the design of the bioreactor system and the optimization of process parameters for scale-up, using the statistical design of experiments (DoE). Fifth, the use of historical data and advanced mathematical models for monitoring and control. Lastly, the development of novel strategies for PHB recovery with a minimum cost of energy and chemical requirement.

1.4 Challenges associated with cyanobacterial bioprocessing

Various challenges are associated with the cyanobacterial process engineering leading therefore to low PHB productivity and the high production costs. The most significant challenges which could be listed are: cyanobacteria are laborious to cultivate in large-scale, they possess small growth rates and are difficult to harvest. Strain improvement using genetic modification tools may lead to more fitness and higher growth rates. So far, a very few examples of cyanobacterial genetic modification has shown improvement in productivity in terms of time-spaced yield or specific rates. PHB production in cyanobacteria is mainly induced by nutrient limitation, mainly of nitrogen and phosphorus. The loss in photosynthetic efficiency under nutrient limitation will reduce the amount of carbon and energy that can be accumulated as PHB. Preventing the loss in photosynthetic efficiency may result in higher PHB productivity since there will be

more energy available in the form of ATP and NADPH to be stored as PHB. Another challenge associated with cyanobacteria as a production system is the PHB recovery, the later issue adversely affects the overall process economy. Also, cyanobacterial PHB is easily degraded as the cultivation conditions slightly fluctuate, therefore, an alternative way to improve the PHB productivity in cyanobacteria would be to knockdown the PHB catabolic genes. The other advantage of blocking PHB catabolism would also be the reduction of PHB degradation during dark-night cycles.

1.5 Kinetics and physiology of cyanobacterial PHB synthesis

Even though the mechanism by which PHB is produced in cyanobacteria is not completely understood, the main accepted hypothesis is that when cyanobacteria are exposed to unfavorable growth conditions, like nitrogen and phosphorus limitation, the excess energy or the disproportion in the ratio of carbon: nitrogen or NADPH: ATP is effectively directed towards carbon reserves such as glycogen and PHB synthesis as reducing equivalents (De Philippis et al., 1992). In some cyanobacterial species, atmospheric carbon dioxide is fixed via the Calvin-Benson cycle, producing carbon constituents; 3-phosphoglycerate for production of metabolites such as glycogen and PHB (Wang et al., 2013). This metabolite is later utilized to provide the necessary carbon backbones for biosynthesis of proteins and other metabolites required for cell growth when essential nutrients are available (Hauf et al., 2015). PHB is synthesized in three biosynthetic steps where acetoacetyl-CoA is formed from two molecules of acetyl-CoA by the enzyme β-ketothiolase (encoded by phaA) (Hauf et al., 2015). In the second step, PhaB (encoded by phaB) reduces acetoacetyl-CoA to hydroxybutyryl-CoA, utilizing NADPH as the electron donor (Taroncher-Oldenburg et al., 2000). In the final step of biosynthesis, 3-hydroxybutyryl-CoA is polymerized to PHB by a PHA synthase, comprising of PhaC and PhaE (Hauf et al., 2015; Hein et al., 1998).

1.6 Strain improvement in cyanobacteria

In industrial biotechnological processes, commonly, wild-type strains are improved using methods such as metabolic engineering, mutation and selection, and breeding. Enhancement of PHB productivity in cyanobacteria using various methods such as genetic modification has been widely practiced. Most cyanobacterial studies on metabolic engineering and PHB biosynthesis have been conducted with a limited number of model strains, of which *Synechocystis* sp. PCC 6803 is the most widely studied species for cyanobacterial research. The analysis, which has been done for decades on photosynthesis and the genome annotations, has resulted in a wide range of metabolic engineering tools and extensive biological insight for this species (Koksharova & Wolk, 2002; Wilde & Dienst, 2011). Efforts to overcome bottlenecks in PHB

synthesis pathways, including upregulation of native biosynthetic genes for PHB production namely phaA, B, C and E in *Synechocystis* sp. PCC 6803 in spite of a two-fold higher PHB content (26 % DCW) (Khetkorn et al., 2016), could not yield higher productivity in terms of produced PHB per unit of time and reactor volume. Also the heterologous expression of *Cupriavidus necator* (Betaproteobacteria) PHB synthesis genes in *Synechocystis* sp. PCC 6803 (Sudesh et al., 2002) showed increased activity of PHB synthase but was not associated with a significant increase in PHB levels. Conversely, lower expression of PHB synthesis genes has been reported in genetically engineered *Synechocystis* sp. PCC 6803 with high PHB accumulation (Lau et al., 2014). These observations suggest that PHB synthesis is not only regulated by the levels of enzymes of PHB synthesis, but by other factors such as the intracellular metabolite concentrations and redox levels (Dutt & Srivastava, 2017). Since the directed approaches used so far could not lead to successful enhancement of PHB productivity in cyanobacteria, the application of an alternative technique seems crucial.

Mutagenesis, as a substitute approach to genetic engineering, has been used successfully to obtain improved bacterial strains as production hosts in biotechnology (Galanie et al., 2013). As an alternative approach to genetic engineering, random mutagenesis can be done for the generation of a mutant library with improved phenotypes. Mutagenesis can be done by exposing the cells of interest to a mutagenic source in order to induce random mutations into the genome. This can, for instance, completely knock-out a gene function (Jaeger, 2015) or increase enzymatic activity. UV-irradiation is the most frequently used mutagen, which leads to transversion in the genome. Furthermore, ethidium bromide and ethyl-methanesulfonate are used as chemical mutagens (Jaeger, 2015; Lee et al., 2014). A major disadvantage of using random mutagenesis is the need for intensive screening to select the mutant with desired phenotypes.

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2) Thesis outline

Cyanobacterial PHB production using sustainable resources CO_2 and sunlight has the advantage of reducing the production costs and bringing reality to industrial production of this biodegradable polymer. Alternatively, the little PHB productivity and the slow growth of cyanobacteria impairs the commercialization and production on an industrial scale. Hence, a scientific and systematic approach is needed to facilitate an understanding of the whole process, allowing for optimization and economically feasible production.

The aim of this thesis is to use a proper, quantitative, time-resolved approach to assess the photosynthetic PHB production in cyanobacteria. On the example of *Synechocystis* sp. PCC 6714, the PHB production process was tuned using different media limitations and cultivation parameters and the yields and kinetics were characterized under defined and controlled conditions of the photobioreactor. Random mutagenesis approach was used to produce more efficient and fit strains with superior characteristics namely increased specific growth rate, higher CO_2 uptake rate, and greater yield and specific polymer productivity. The in-depth characterization of high PHB-yielding mutant and the wild-type parent strain helped for a physiological understanding of PHB and glycogen accumulation mechanism and to define targets for the future strain improvement strategies. Also, the quantitative and step-by-step media limitations aided the knowledge generation for designing of a simple and scalable bioprocess for production in industrial range. Furthermore, the analysis of the photosynthetically produced polymer showed the potential of the PHB produced in cyanobacteria for various extended applications. The ideas of technology- transfer from laboratory to the industry may result in more successful attempts for pilot-scale photosynthetic PHB production.

3) Structure of the thesis

This is a cumulative thesis comprising of four already published peer-reviewed papers and a manuscript under the revision. The thesis is divided into four parts representing a quantitative approach for assessment of photosynthetic PHB production in cyanobacteria, namely 1) State of the art and bioprocess engineering aspects of sustainable PHB production in cyanobacteria 2) Cyanobacteria as production platform; strain screening and improvement 3) Bioprocess optimization and understanding of physiological responses to media limitations 4) assessment of product quality and ideas for technology-transfer for production in industrial scale.

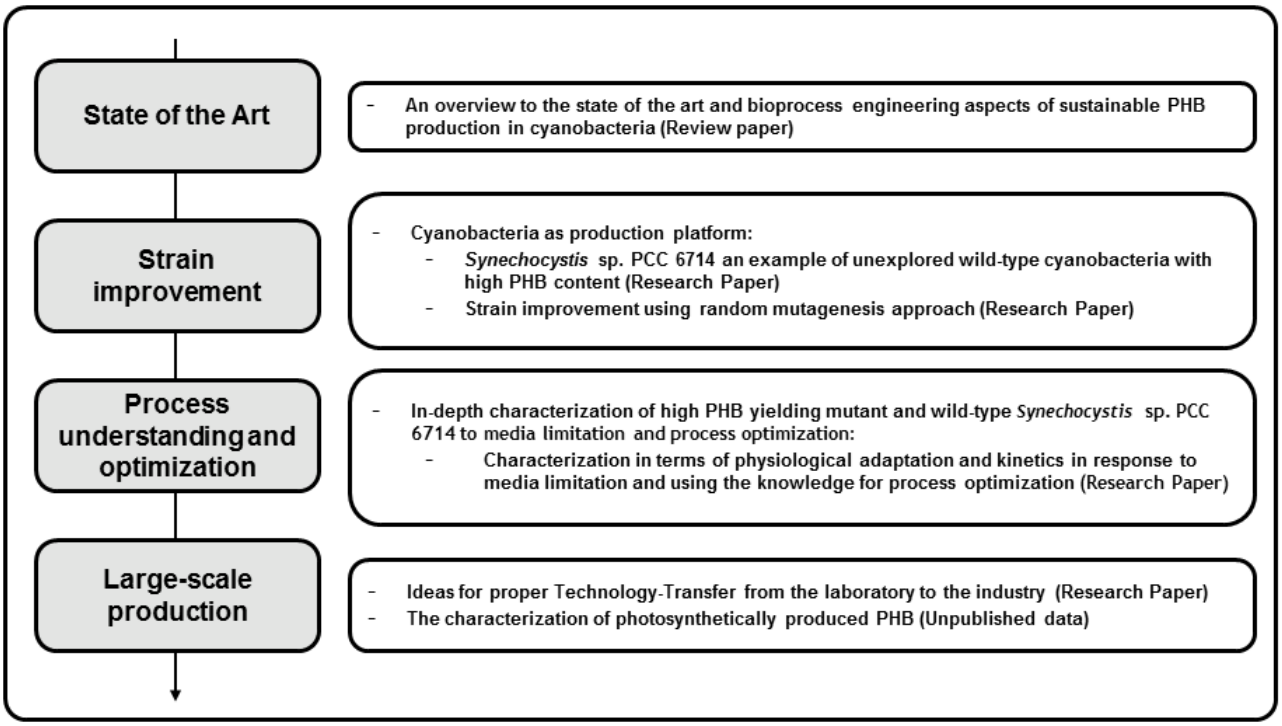

Figure 3.1: Represents an overview to the structure of the thesis

The first part provides an introduction to the state of the art of cyanobacterial bioprocess engineering and the challenges associated with the commercialization of photosynthetically produced PHB.

The second part deals with cyanobacterial strain selection and strain improvement. In this session the cultivation of *Synechocystis* sp. PCC 6714 under controlled and defined conditions

of a photobioreactor is provided in order to emphasize the potential of an unexplored wild-type cyanobacterial strain for phototrophic production of PHB. To obtain improved cyanobacteria an alternative, a non-GMO approach using random mutagenesis is demonstrated. The study of the high PHB-yielding mutant is done in terms of growth and productivities and the characterization is performed using phenotyping assays and quantitative PCR. The evaluation of the genome of the best mutant, MT_a24 is performed in details.

The third part deals with the in-depth characterization of the best mutant, MT_a24, and the parent strain, wild-type *Synechocystis* sp. PCC 6714. The two aforementioned strains are characterized in terms of physiology and the kinetics with respect to the nutrient availability and the cultivation mode and the knowledge is used for designing of a simple and scalable process for production in commercial scale.

The final part deals with the quality assessment of the photosynthetically produced PHB and the ideas for the technology-transfer from the laboratory to the industry.

Part II

Results

4) Finding and achievements

The following chapter provides an overview to the state of the art of cyanobacterial bioengineering, the challenges associated with photosynthetic PHB production, the ideas, and suggestions to overcome the bottlenecks in the production process and lists the corresponding manuscripts. In addition, the impact of accomplished tasks on the industrial production of PHB is emphasized.

Bioprocess engineering of PHB production using cyanobacteria as a cell factory

Challenges: Generally, the productivity of phototrophically produced PHA from cyanobacteria is much lower than in the case of heterotrophic bacteria. Great effort has been focused to increase the polymer content in cyanobacteria, mainly by the development of optimized strains and more efficient cultivation and recovery processes. Minimization of the PHA production cost can only be achieved by considering the design and a complete analysis of the whole process.

State of the art: Current industrial PHA production processes rely mostly on the availability of agricultural resources, which are unsustainable and sometimes have an ecological footprint. In the case of cyanobacterial PHA production research has mainly focused on genetic engineering to increase productivity, which mainly reports as a higher percentage of dry cell weight content. The studies have rarely reported an increase in photosynthetic efficiency or an increase in the specific growth rates or production rates.

Findings: With respect to commercialization and scale-up of the cyanobacterial PHA production, the view of the whole processes needs to be considered. More attention towards sustainable and viable upstream processing may help to reach an economic PHA production point. Photosynthetic PHA production, from an economic point of view, will only make sense if a continuous process can be achieved, especially using waste streams as the co-substrate and also for the media preparation. The process can then be coupled with the bioremediation of agricultural and industrial effluents. Thus far, some wild-type and improved cyanobacterial strains are reported with PHB content which is mostly cultivated under controlled, defined, and sterile lab conditions. For production in industrial scale which is done under unsterile

conditions, only *Nostoc moscorum* as an example is reported. Other strains are not tested or can hardly tolerate the harsh outdoor conditions. Although the importance of optimized media and cultivation conditions on PHA productivity is emphasized, sustainable and viable commercial processes conducted under unsterile conditions using waste streams and open systems are required. Research needs to focus on screening for more robust strains, such as wastewater born mixed-cultures that can tolerate fluctuations in cultivation conditions like pH, temperature, salinity, and media composition. Furthermore, the durable strains for which production of PHB is associated with biomass growth and therefore the time-resolved productivity can be improved.

Impacts of the work: An overview of the state of the art for the cyanobacterial bioprocess engineering and the challenges associated with photosynthetic PHB production is listed. The impact of considering the analysis and design of the whole process for the economically feasible production of photosynthetic PHB is emphasized.

Citation: Kamravamanesh Donya, Lackner Maximilian, Herwig Christoph. Bioprocess engineering aspects of sustainable Polyhydroxyalkanoate production in cyanobacteria. Journal of Bioengineering 2018; 5(4).111.

My contribution: I set up the concept of the manuscript with Christoph Herwig and wrote the paper.

Cyanobacteria as production platform; strain selection and improvement

Challenges: As already discussed PHB production in cyanobacteria from an economic point of view has two major drawbacks: little productivity and slow growth. In order to promote photosynthetic PHB production on an industrially relevant scale, the productivity needs significant improvement and industrially relevant cyanobacterial strains have to be developed. Current research, mainly focuses on increasing the PHB content in terms of the dry cell weight and not time-resolved productivity. The productivity is defined as the amount of PHB produced by unit volume in unit time.

State of the art: In spite of all the efforts done, so far, to increase PHB accumulation in cyanobacteria a very few reports have shown an actual improvement in the photosynthetic PHB production process, while there are various challenges associated with the cultivation, engineering, and scale-up of autotrophic cyanobacterial biomass. A quantitative, time-resolved approach needs to be considered in order to facilitate the understanding and full characterization of the production process.

Findings: As an example of unexplored, wild-type cyanobacterial strain, the *Synechocystis* sp. PCC 6714 was cultivated under optimized cultivation parameters and controlled, defined conditions of a photobioreactor using various nitrogen sources. A quantitative approach was used for understanding the PHB accumulation in response to different media limitations. The combined effects of nitrogen and phosphorus limitation led to an increase in polymer productivity from CO_2, showing the highest volumetric production rate of 59 $\pm$ 6 mg L^{-1} day^{-1}. Multivariate experimental design and quantitative bioprocess development methodologies were used to identify the key cultivation parameters for PHB accumulation.
The random mutagenesis approach was used for the production of high PHB-yielding mutants of *Synechocystis* sp. PCC 6714 and the selection of strains was based on superior growth and PHB content induced by nitrogen and phosphorus limitation. The best mutant, MT_a24 showed increased specific growth rates, improved CO_2 uptake rate and a 2.5 fold increase in polymer content obtaining values of 37 ± 4 % dry cell weight PHB. Corresponding to the maximum volumetric PHB productivity of 134 ± 13 mg L^{-1} day^{-1}. Phenotyping assays showed the better fitness of the strain MT_a24. Genome sequencing showed that UV-mutagenesis treatment resulted in a point mutation in the ABC-transport complex, phosphate-specific transport system protein A (pstA).

Impacts of the work: The cyanobacterial strain *Synechocystis* sp. PCC 6714 was introduced for the first time as a phototrophic PHB producer. The potential of random mutagenesis for the production of high PHB producing cyanobacteria from CO_2 was demonstrated. Also,

the potential of the mutant, MT_a24 for industrial production of PHB and also for carbon capture from the atmosphere and point sources was demonstrated. Finally, future targets for genetic engineering in cyanobacteria with an aim to increase PHB productivity from CO_2 were identified.

Citation: Kamravamanesh Donya, Pflügl Stefan, Nischkaur Winfried, Limbeck Andreas, Lackner Maximilian, Herwig Christoph. Photosynthetic poly-β -hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714. Journal of applied microbiology and biotechnology express. 2017; 7:143.

My contribution: I set up the concept of the manuscript with Christoph Herwig and Stefan Pflügl, performed the experiments, analyzed the data and wrote the paper.

Citation: Kamravamanesh Donya, Kovacs Tamas, Pflügl Stefan, Druzhinina Irina, Kroll Paul, Lackner Maximilian, Herwig Christoph. Increased poly-β -hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: Mutant generation and characterization. Bioresource Technology 266. 2018: 34-44.

My contribution: I set up the concept of the manuscript with Christoph Herwig and Stefan Pflügl, performed the experiments with the support of Tamas Kovacs, analyzed the data and wrote the paper.

Bioprocess optimization and understanding of physiological responses

Challenges: Cyanobacteria accumulate PHB under limiting growth conditions, making, therefore, the PHB production in a two-step process comprising of a growth phase followed by a nutrient limitation phase. In order to maintain high PHB content in a continuous mode of operation and to establish an easily scalable and controllable bioprocess for photosynthetic production of PHB, media optimization has to be done.

State of the art: Glycogen biosynthetic pathway in cyanobacteria is considered as a competing pathway, showing a double function: one as a carbon reserve and also as a buffering substance. Recently photosynthetic production of glycogen has gained great importance as it can be converted to value-added compounds such as bioethanol using yeast fermentation. However, the impact on the increase in glycogen content has not been discussed so far.

Findings: While the mutant MT_a24 showed a significantly higher PHB productivity the subsequent goal was the understanding of the supremacy of the mutation and therefore using the knowledge for improvement and stabilization of the production process. In order to provide an easily scalable and alternative approach to the normally done two-step process -comprising of growth phase and limitation phase- one-step cultivation was optimized. The multivariate experimental design approach was used for the optimization of the one-step, self-limiting media. During one-step cultivation of MT_a24 with optimized media 30 ± 4 % (DCW) corresponding to 1.16 g L^{-1}, PHB was obtained. Using pulse experiments it was demonstrated that phosphate is the key driver of glycogen synthesis in *Synechocystis* sp. PCC 6714 and it could be used to boost glycogen productivity. The maximum glycogen content acquired was 2.6 g L^{-1} (76.2 % DCW) for mutant MT_a24 using phosphate feeding and carbon dioxide as the only carbon source.

Impacts of the work: Bioprocess improvement tools have a great advantage to increase sustainable carbohydrate production in cyanobacteria. The work showed the potential of glycogen enriched MT_a24 for bio-refinery and as feedstock for the production of energy and value-added compounds, such as PHB. The transient phosphorus availability was demonstrated to improve carbohydrate production efficiency mainly in terms of glycogen production in mutant MT_a24. Using media optimization, a one-step easily scalable bioprocess was optimized for industrial production of PHB.

Citation: Kamravamanesh Donya, Slouka Christoph, Limbeck Andreas, Lackner Maximilian, Herwig Christoph. Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: bioprocess understanding and evaluation of productivities. Bioresource Technology 273 (2019) 277-287.

My contribution: I set up the concept of the manuscript, performed the experiments, analyzed the data and wrote the whole paper.

Scale-up challenges and ideas for technology-transfer for production in industrial scale

Challenges: The use of PHB derived from cyanobacteria is not economically feasible due to high production costs.

State of the art: Although extensive research has been directed towards optimization of the strain, media and the process, yet there is scarce knowledge on the performance and viability of large-scale PHB production lines exploiting cyanobacteria.

Findings: The technology-transfer in biopharmaceutical industry and biotechnology-based manufacturing is necessary to elucidate crucial information to be transferred from R & D to pilot-production and for development of existing process to the production in industrial-scale.

Impacts of the work: The knowledge gap between the laboratory and industry is filled for sustainable and renewable production of PHB from CO_2.

Citation: Kamravamanesh Donya, Kiesenhofer Daniel, Fluch Silvia, Lackner Maximilian, Herwig Christoph. Scale-up challenges and requirement of technology-transfer for cyanobacterial poly (3-hydroxybutyrate) production in industrial scale. Under revision at the Journal of Bioresource Technology.

My contribution: I set up the concept of the manuscript with Christoph Herwig, analyzed the data and wrote the whole paper.

Quality assessment of photosynthetically synthesized poly (3-hydroxybutyrate)

Challenges: The existing pessimistic views on the material characteristics of PHB makes the need to advance the study and characterize of the cyanobacterial derived polymer.

State of the art: PHB with a high crystallinity represents properties similar to synthetic polyesters and also to polyolefins such as polypropylene. However, the low elongation and break and the brittleness of PHB are limitations which can be overcome by using other PHA, blends of copolymers.

Findings: The Fourier Transform Infrared spectroscopy (FTIR) showed the characteristic signals expected for the phototrophic PHB. Differential Scanning Calorimetry (DSC) used to determine the thermal characterization of PHB showed melting temperature of 151,42 °C and 73,11 °C for the crystallization process. From the Gel Permeation Chromatography (GPC) the molecular weight of cyanobacterial PHB was determined to be 1051900 g mol^{-1}.

Impacts of the work: PHB can be produced using the mutant cyanobacterial strain MT_a24 from sustainable resources CO_2 and sunlight and the material has the potential to be utilized in extended applications.

Citation: Kamravamanesh Donya, Krampl Margit, Itzinger Regina, Paulik Christian, Chodak Ivan, Lackner Maximilian and Herwig Christoph, Quality assessment of photosynthetically synthesized poly (3-hydroxybutyrate) using a randomly mutated strain of *Synechocystis* sp. PCC 6714, Unpublished data

My contribution: I designed the study with Maximilian Lackner. Extracted the material with the help of Margit Krampl and structured and wrote 50 % of the manuscript.

5) Manuscripts

5.1 Bioprocess engineering of PHB production using cyanobacteria as a cell factory

5.1.1 Bioprocess engineering aspects of sustainable polyhydroxyalkanoate production in cyanobacteria

Abstract: Polyhydroxyalkanoates (PHAs) are a group of biopolymers produced in various microorganisms as carbon and energy reserve when the main nutrient, necessary for growth, is limited. PHAs are attractive substitutes for conventional petrochemical plastics, as they possess similar material properties, along with biocompatibility and complete biodegradability. The use of PHAs is restricted, mainly due to the high production costs associated with the carbon source used for bacterial fermentation. Cyanobacteria can accumulate PHAs under photoautotrophic growth conditions using CO_2 and sunlight. However, the productivity of photoautotrophic PHA production from cyanobacteria is much lower than in the case of heterotrophic bacteria. Great effort has been focused to reduce the cost of PHA production, mainly by the development of optimized strains and more efficient cultivation and recovery processes. Minimization of the PHA production cost can only be achieved by considering the design and a complete analysis of the whole process. With the aim on commercializing PHA, this review will discuss the advances and the challenges associated with the upstream processing of cyanobacterial PHA production, in order to help the design of the most efficient method on the industrial scale.

This manuscript is published in Journal of Bioengineering (Basel).

Kamravamanesh Donya, Lackner Maximilian, Herwig Christoph. Bioprocess engineering aspects of sustainable Polyhydroxyalkanoate production in cyanobacteria. Journal of Bioengineering 2018; 5(4).111.

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Review

Bioprocess Engineering Aspects of Sustainable Polyhydroxyalkanoate Production in Cyanobacteria

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Received: 22 November 2018; Accepted: 15 December 2018; Published: 18 December 2018

Abstract: Polyhydroxyalkanoates (PHAs) are a group of biopolymers produced in various microorganisms as carbon and energy reserve when the main nutrient, necessary for growth, is limited. PHAs are attractive substitutes for conventional petrochemical plastics, as they possess similar material properties, along with biocompatibility and complete biodegradability. The use of PHAs is restricted, mainly due to the high production costs associated with the carbon source used for bacterial fermentation. Cyanobacteria can accumulate PHAs under photoautotrophic growth conditions using CO_2 and sunlight. However, the productivity of photoautotrophic PHA production from cyanobacteria is much lower than in the case of heterotrophic bacteria. Great effort has been focused to reduce the cost of PHA production, mainly by the development of optimized strains and more efficient cultivation and recovery processes. Minimization of the PHA production cost can only be achieved by considering the design and a complete analysis of the whole process. With the aim on commercializing PHA, this review will discuss the advances and the challenges associated with the upstream processing of cyanobacterial PHA production, in order to help the design of the most efficient method on the industrial scale.

Keywords: polyhydroxyalkanoate (PHA), bioprocess design; carbon dioxide; cyanobacteria; upstream processing

1. Introduction

Petroleum-based polymers are relatively inert, versatile, and durable; therefore, they have been used in industry for more than 70 years [1]. However, they bear negative properties such as CO_2 emissions from incineration, toxicity from additives, and accumulated toxic substances in the environment, particularly in marine as microplastics, recalcitrance to biodegradation, and massive waste accumulation into the marine environment and the landfills [2,3]. With the limited fossil fuel resources and the environmental impact associated with the products, the research for an alternative seems essential in order to reduce our dependencies on non-renewable resources [4,5].

Biodegradable polymers, due to their eco-friendly nature, offer one of the best solutions to environmental problems caused by synthetic polymers [5]. Polyhydroxyalkanoates (PHAs) are a class of naturally occurring polymers produced by microorganisms [1,6,7], among which poly (3-hydroxybutyrate) (PHB) is the most studied biodegradable polymer that accumulates in bacteria in the form of inclusion bodies as carbon reserve material when cells grow under stress conditions [5,8]. PHB with a high crystallinity represents properties similar to synthetic polyesters and also to polyolefins such as polypropylene [6,9,10]. In addition, due to biocompatibility and biodegradability,

PHB possesses extensive interesting functions and can replace fossil-based plastics in many applications [7]. However, the low elongation and break and the brittleness of PHB are limitations that can be overcome using other PHA, like blends of copolymers such as polyhydroxyvalerate (PHV) and poly (3-hydroxybutric acid-co-3-hydroxyvaleric acid) (PHBV). The copolymer can either be directly biosynthesized under varying cultivation conditions or be chemically produced in vitro. Apart from short-chain length PHA, there are medium- and long- chain-length polymers which can help to tailor the material properties [11]. The strategies to overcome these limitations are studied in various wild-type and recombinant cyanobacteria, reviewed by Lackner et al. and Balaji et al. [11,12].

Today, PHB is commercially produced by heterotrophic bacteria, such as *Cupriavidus necator* (*C. necator*), and recombinant *Escherichia coli* (*E. coli*) [6,13,14]. High production cost, when compared with petroleum-based polymers, is one major challenge for extensive production and commercialization of PHB [5]. Major contributors to the overall cost being the expensive substrates, continuous oxygen supply, equipment depreciation, high energy demand, and chemicals used for downstream processing [15–17].

Attention has been focused to reduce the production cost, mostly by selecting more economically feasible and efficient carbon substrates for PHB production such as whey, hemicellulose, sugar cane, agricultural wastes, and molasses [5,18–20]. In this context, PHB production using cyanobacteria from more sustainable resources, such as CO_2, has gained importance. Cyanobacteria are an ideal platform for the production of biofuels and bulk chemicals through efficient and natural CO_2 fixation [21].

Other reviews have mainly discussed the potential of cyanobacteria for PHA production, cultivation conditions, and cyanobacterial metabolism, as well as the applications and industrial prospects of the synthesis of this biopolymer [22–26]. Minimization of the PHA production cost can only be achieved by considering the design and a complete analysis of the whole process [27]. In this work, the authors will discuss the bioprocess engineering aspects that focus on upstream processing and advances of sustainable PHA production from cyanobacteria, concentrating primarily on the unit operations of the upstream processing. The authors believe that a proper-time resolved quantification of the process will aid in a better understanding for process manipulation and optimization of industrial production.

2. Cyanobacteria: the Future Host in Biotechnology

Cyanobacteria are gram-negative bacteria with a long evolutionary history and are the only prokaryotes capable of plant-like oxygenic photosynthesis [28]. Unlike heterotrophic organisms, cyanobacteria require only greenhouse gas CO_2 and sunlight, along with minimal nutrients for growth, eliminating the cost of carbon source and complex media components [28]. Cyanobacteria are equipped with superior photosynthetic machinery, showing higher biomass production rates compared to plants and can convert up to 3–9% of the solar energy into biomass [28,29]. Moreover, in contrast to plants, cultivation of cyanobacteria requires less land area, therefore cyanobacteria do not compete for arable land used for agriculture [30]. Some cyanobacteria can produce PHB, when essential nutrients for growth, such as nitrogen and phosphorus, is limiting. From an economic point of view, however, photosynthetic PHB production in cyanobacteria has two major disadvantages: little productivity and slow growth [25]. Therefore, in order to promote photosynthetic PHB production on an industrially relevant scale, the productivity needs to improve significantly. Productivity is defined as the amount of PHA produced by unit volume in unit time [27]. In spite of all the efforts done, so far, very few reports have shown an actual improvement in the cyanobacterial PHB production process, while there are various challenges associated with cultivation, engineering and large-scale production of autotrophic cyanobacterial biomass.

3. Challenges in Cyanobacterial Bioprocess Technology

Figure 1 represents a bioprocess development chain for cyanobacterial PHA production consisting of strain and bioprocess developments and the downstream processing. The workflow shows the strain selection, strain improvement, process understanding, and strategies for scale-up and then down-stream processing, representing the separation and purification of the final product.

In order to obtain an optimal scalable bioprocess and in-depth understanding of the bioprocess kinetics, the following points have to be known. First, the holistic knowledge of enzymatic and metabolic pathways of the PHB biosynthesis in cyanobacteria. Second, the selection or development of the optimal strain with maximum productivity. Third, the selection of inexpensive substrate and optimization of media components for the particular production strain. Fourth, the design of the bioreactor system and the optimization of process parameters for scale-up, using the statistical design of experiments (DoE). Fifth, the usage of past data and advanced mathematical models for monitoring and control. Lastly, the development of novel strategies for PHB recovery with a minimum cost of energy and chemical requirement. This review will mainly focus on the bioprocess engineering aspects of photosynthetic PHB production providing an overview of the PHB production process chain starting from a single cell.

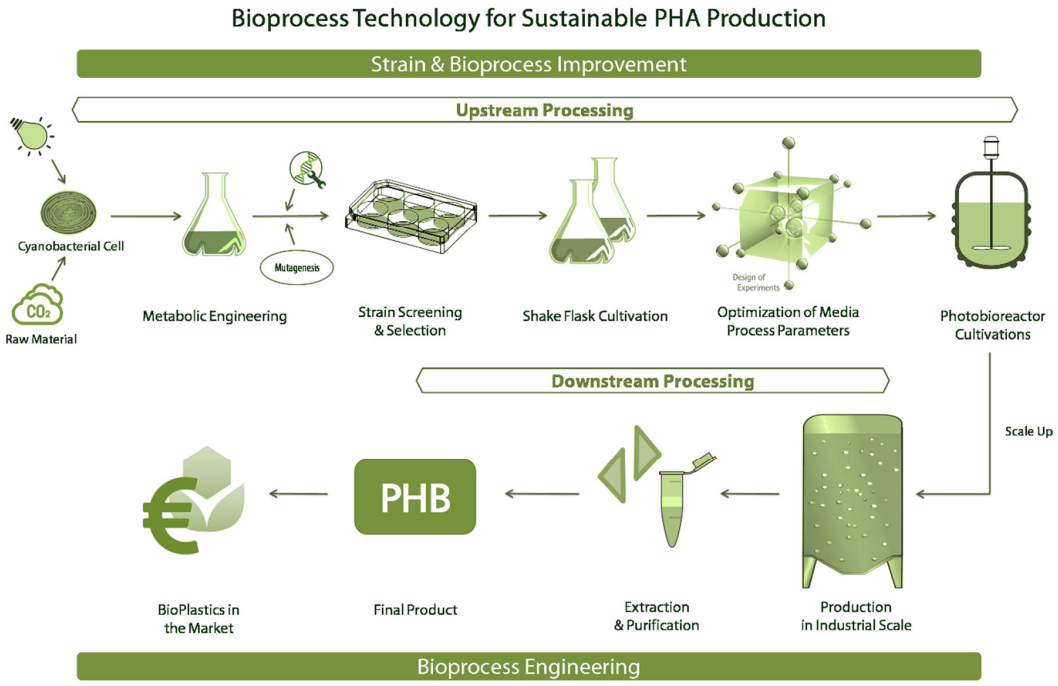

Figure 1. Represents the work-flow in bioprocess technology for the production of polyhydroxyalkanoates (PHAs) using cyanobacteria as the host system.

3.1. Process Design and Optimization

3.1.1. Existing Wild-Type Strains and Their Reported PHB Content

Cyanobacteria are indigenously the only organisms that produce PHA biopolymers using oxygenic photosynthesis [24]. Cyanobacteria grow mainly under autotrophic conditions, nevertheless, supplementation of sugars or organic acids in some species increases growth and PHB accumulation [24], which contributes to the production cost. To date, a few cyanobacterial strains have been identified for photosynthetic PHB accumulation. Table 1 presents the wild-type cyanobacterial strains, their PHB content in dry cell weight (DCW), and the carbon source used for the production.

As is indicated in Table 1, the PHB production process using CO_2 as the only carbon source shows a lower product content than using organic acids or sugars as substrate.

Table 1. Examples of wild-type cyanobacterial strains, with reported poly (3-hydroxybutyrate) (PHB) content, the carbon source, and growth conditions used for the production of the polymer.

Cyanobacteria	PHB Content (% DCW)	Substrate	Production Condition	Polymer Composition	Reference
Synechocystis sp. PCC 6803	38	Acetate	P limitation and gas exchange limitation	PHB	[31]
Synechocystis sp. PCC 6714	16	CO_2	N and P limitation	PHB	[32]
Spirulina platensis	6.0	CO_2	Not given	PHB	[33]
Spirulina platensis UMACC 161	10	acetate and CO_2	N starvation	PHB	[34]
Spirulina maxima	7–9	CO_2	N and P limitation	PHB	[35]
Gloeothece sp. PCC 6909	9.0	acetate	Not given	Not specified	[36]
Nostoc moscorum Agardh	60	acetate and valerate	N deficiency	PHB-co-PHV	[37]
Nostoc moscorum	22	CO_2	P starvation	PHB	[38]
Alusira fertilisima CCC444	77	fructose and valerate	N deficiency	PHB-co-PHV	[39]
Alusira fertilisima CCC444	85	citrate and acetate	P deficiency	PHB	[40]
Synechocystis PCC 7942	3	CO_2	N limitation	PHB	[41]
Synechocystis PCC 7942	25.6	acetate	N limitation	PHB	[41]
Synechocystis sp. CCALA192	12.5	CO_2	N limitation	PHB	[42]
Anabaena Cylindrica	<0.005	CO_2	Balanced growth	PHB	[43]
Anabaena cylindrica	2.0	propionate	N limitation	PHB + PHV	[43]
Synechococcus elongatus	17.2	CO_2 and sucrose	N deficiency	Not specified	[44]
Caltorix scytonemicola TISTR 8095	25	CO_2	N deficiency	PHB	[45]

[1] gas exchange limitation = limitation of gas transfer to the culture vessel. [2] N = nitrogen. [3] P = phosphorus.

3.1.2. More Competent Cyanobacterial Cell Lines

Cyanobacteria are considered a sustainable and alternative host for PHB production due to their photoautotrophic nature [46]. Despite the fact that cyanobacterial PHB has been the subject of research for many years, it has not found its way to the market. One of the main challenges for cyanobacterial products to enter the market is that cyanobacterial strains are not yet optimized as cell factories for industrial processes. Intensive research has been done over the past 20 years for cyanobacterial strain improvement, research that has aimed to increase PHB productivity, mainly by overexpression of PHB biosynthetic genes. However, these attempts have rarely shown success regarding increased volumetric or specific polymer content for commercial production of cyanobacterial PHB. Recently, Katayama et al. reviewed the production of bioplastic compounds using genetically modified and metabolically engineered cyanobacteria [47]. In this study, we provide a list of genetically modified cyanobacteria with their PHB content and the tools used for the metabolic engineering of the strain.

3.1.3. Genetic Engineering of Cyanobacteria for PHB Production

Being prokaryotic, cyanobacteria possess a relatively simple genetic background which eases their manipulation [48]. Most cyanobacterial studies on metabolic engineering and PHB biosynthesis have been conducted with a limited number of model strains, of which *Synechocystis* sp. PCC 6803 is the most widely studied species for cyanobacterial research. The research, which has been done for decades on photosynthesis and the genome annotations, has resulted in a wide range of metabolic engineering tools and extensive biological insight for this species [48,49]. PHB production in cyanobacteria occurs mainly via three biosynthetic steps, where two molecules of acetyl-CoA form one molecule of acetoacetyl-CoA using the enzyme 3-ketothiolase encoded with the *phaA* gene [50]. Later, acetoacetyl-CoA is reduced by PhaB to hydroxybutyryl-CoA, utilizing NADPH as an electron donor [51]. In the end, the PHA synthase comprises of PhaC and PhaE polymerizes (R)-3-hydroxybutyryl-CoA to PHB [50,52]. Table 2 summarizes the efforts to overcome the bottlenecks in PHB biosynthetic pathway in cyanobacteria.

Table 2. Strategies to increase PHB biosynthesis yield.

Cyanobacterial Strain (Recombinant)	PHB Content (% DCW)	Genetic Tool Used	Production Conditions	References
Synechococcus sp. PCC 7942	1.0	Defective in glycogen synthesis	CO_2	[53]
Synechococcus sp. PCC 7942	26	Introducing PHA biosynthetic genes from *C. necator*	Acetate and nitrogen limitation	[41]
Synechocystis sp. PCC 6803	26	Overexpression of native *pha* genes	CO_2 and nitrogen deprivation	[46]
Synechocystis sp. PCC 6803	11	Introducing PHA biosynthetic genes from *C. necator*	Acetate and nitrogen limitation	[54]
Synechocystis sp. PCC 6803	14	Overexpression of PHA synthase	Direct photosynthesis	[55]
Synechocystis sp. PCC 6803	12	Increasing acetyl-CoA levels	CO_2	[56]
Synechococcus sp. PCC 7002	4.5	Introduction of GABA Shunt	CO_2	[57]
Synechocystis sp.	35	Optimization of acetoacetyl-CoA reductase binding site	CO_2	[58]
Synechocystis sp. PCC 6803	7.0	Transconjugant cells harboring expression vectors carrying *pha* genes	CO_2	[59]

Metabolic engineering of cyanobacteria with the aim to increase PHB content was also done by introducing the PHA synthase gene from *C. necator* into *Synechhocytis* sp. PCC 6803 [54]. The resulting recombinant *Synechhocytis* sp. PCC 6803 showed increased PHA synthase activity; the total PHB content, however, did not increase [47,54]. For cyanobacterial strain *Synechocystis* sp. PCC 6803, up to 35% (DCW) PHB was obtained using phaAB overexpression and 4 mM acetate [46]. However, the specific production rates in this case also did not show a significant improvement either. Recently, the overexpression of the acetoacetyl-CoA reductase gene in *Synechocystis* was found to increase the productivity of R-3-hydroxybutyrate from CO_2 to up to 1.84 g L^{-1} [58]. The highest volumetric productivity reported in this case was 263 mg L^{-1} d^{-1}.

3.1.4. Randomly Mutated Strains with Improved PHB Content

As an alternative approach to genetic engineering, random mutagenesis can be done for the generation of a mutant library with improved phenotypes. Mutagenesis can be done by exposing the cells of interest to a mutagenic source in order to induce random mutations into the genome. This can, for instance, completely knock-out a gene function [60] or increase enzymatic activity. UV irradiation is the most frequently used mutagen, which leads to transversion in the genome. Furthermore, ethidium bromide and ethyl-methanesulfonate are used as chemical mutagens [60,61]. A major disadvantage of using random mutagenesis is the need for intensive screening to select the mutant with desired phenotypes. The cyanobacterial strain *Synechocystis* sp. PCC 6714 has a great potential as photosynthetic PHB production organism. It has shown up to 17% (DCW) PHB content under nitrogen and phosphorus limiting conditions [32]. In addition to PHB, the strain also accumulates glycogen during the early phase of nitrogen limitation [62]. The random mutagenesis approach used for *Synechocystis* sp. PCC 6714 showed an increase in productivity of up to 2.5-folds resulting in 37 ± 4% (DCW) PHB for the best mutant [63]. The UV-mutation lead to an amino acid change in the phosphate system transport protein (PstA), resulting in higher efficiency of photosynthesis and CO_2 uptake rate for the mutant MT_a24 [63].

3.1.5. CRISPR/Cas Based Genome Editing in Cyanobacteria

Cyanobacteria are promising platforms for the production of biofuels and bio-based chemicals, however, the metabolic engineering of cyanobacteria poses various challenges [64]. CRISPR/Cas technology has enabled genome modification of cyanobacteria with gene substitution, marker-less point mutations, and gene knockouts and knock-ins with improved efficiency [65]. So far, the CRISPRi system has been used to downregulate the production of PHA and glycogen production in

order to increase fluxes towards other carbon storage compounds of interest [66], such as succinate [64]. However, the CRISPRi based gene editing to overexpress PHB biosynthetic genes has not been reported. While the CRISPR-based editing allows the creation of marker-less knockouts and knock-ins. Thus, in the future, the cyanobacterial strains produced might be considered commercially sustainable and safe for outdoor cultivations and CO_2 sequestration.

3.2. Process Design and Bioprocess Improvement Strategies

Nutrient deficiency or stress, mainly in terms of nitrogen or phosphorus limitation, stimulates the accumulation of PHB in cyanobacteria. Besides the strain engineering and improvement approach, various reports have discussed other factors, which can facilitate superior growth and productivity in cyanobacteria. Herein, the most important routes for improvement of PHB production in cyanobacteria are listed.

3.2.1. Media and Cultivation Conditions

Like all other bioprocesses, PHB production from cyanobacteria is mainly influenced by the cultivation parameters and nutrient supply. The importance of defined cultivation conditions used to obtain highly productive process for cyanobacteria and microalgae has been previously discussed [32,67,68]. Cyanobacterial growth requires a high concentration of essential nutrients, such as nitrogen, phosphorus, sulfur, potassium, magnesium, iron, and some traces of micromolecules. The supply of nutrients like nitrogen and phosphorus in limiting concentration is important for the production of PHB. Therefore, media optimization plays an important role to maximize the PHB productivity and lower production costs. Regarding optimized nitrogen concentration in the media, it was shown by Coelho et al. that 0.05 g L^{-1} nitrogen in the media results in the production of up to 30.7% (DCW) of PHB in *Spirulina* sp. LEB 18. Further optimization of nitrogen content to 0.22 g L^{-1} in the media increased the PHB content in spirulina sp. LEB 18 to 44.2% (DCW) [69]. However, the impact of nitrogen optimization on the volumetric or specific productivities were not reported in both cases. The optimization of media components, nitrogen, and phosphorus in the case of *Synechocystis* sp. PCC 6714 increased volumetric as well as specific production rates, both in the case of biomass growth and PHB content [62].

Besides media components, other key parameters influencing growth and PHB production in cyanobacteria are cultivation conditions, such as temperature, pH, light intensity, or light/dark cycles. Furthermore, production of the copolymers can be tailor-made by using co-substrates and varying the cultivation conditions, such as temperature and pH [70]. Various studies have used the statistical design of experiments (DoEs) in order to optimize the media as well as the cultivation conditions [31,32,71]. The DoEs are used to minimize the error in determining the influential parameters, allowing systematic and efficient variation of all factors [72]. Table 3 summarizes the cultivation parameters and the nutrient limitation used for cyanobacterial PHB synthesis.

Table 3. Reported cultivation parameters and media limitation used for photoautotrophic PHB production in wild-type cyanobacterial strains.

Cyanobacterial Strain	Limiting Component	Temperature °C	pH	Light Condition	PHB Content % (DCW)	Cultivation Time (days)	Volume (L)	References
Synechocystis sp. PCC 6803	N and P starvation	28–32	7.5–8.5	dark/light cycle	11	10	0.05	[31]
Synechocystis sp.PCC6803	N starvation	30	n.p	light	4.1	7	n.p	[73]
Synechocystis sp.PCC6803	N limitation	28	n.p	18:6	8	30	0.8	[74]
Synechocystis sp. PCC 6714	N and P limitation	28	8.5	light	16.4	16	1	[32]
Synechocystis salina CCALA192	Optimized BG-11 media	n.p	8.5	light	6.6	21	200	[23]
Phormidium sp. *TISTR 8462*	N limitation	28	7.5	light	14.8	12	n.p	[75]
Calothrix scytonemicola TISTR 8095	N deprivation	28	7.5	light	25.4	12	n.p	[75]
Nostoc muscorum	[4]Growth associated	25	8.5	14:10	8.6	21	0.05	[76]
Nostoc muscorum	P depletion	22	n.p	light	10.2	19	n.p	[38]
Spirulina sp. LEB 18	[5]Defined media	30	n.p	12:12	30.7	15	1.8	[77]
Aulosira fertilissima	P limitation	28	8.5	14:10	10	4	0.05	[40]
Anabaena sp.	n.p	25	8	14:10	2.3	n.p	0.1	[78]

[4] n.p = not provided. [5] Optimized BG-11 media = the optimized BG-11 media contains 0.45 g L^{-1} $NaNO_3$ and leads to a self-limitation of the culture. [6] Growth associated = the production of PHA was associated with growth and no media limitation was given. [7] Defined media = concentration of nitrate, phosphate and sodium bicarbonate was optimized.

Two primary challenges of entering cyanobacterial PHB into the market are the concern of the sustainability of the production process and the high production costs of fresh water and nutrients. One solution could be to use waste streams like agricultural effluents with high nitrogen and phosphorus contents. Therefore, the production of the polymer is accompanied by the removal of nutrients from the water. On the other hand, the undefined substrate may raise new challenges that then need to be resolved [79]. Various reports have shown production of cyanobacterial PHB using waste streams. Troschl et al. have summarized the list of cyanobacterial strains cultivated on agro-industrial waste streams and anaerobic digestants to produce PHB [23]. One example is the cultivation of the diazotrophic cyanobacterial strain *Aulosira fertilisima* under a circulatory aquaculture system that resulted in increased dissolved oxygen levels during the cultivation period and the complete removal of nutrients, such as ammonia, nitrite, and phosphate, within 15 days of cultivation, yielding an average PHB content of 80–92 g m^{-3} [80]. This report, along with other previously shown studies [79,81–84], clearly shows the potential of cyanobacterial PHB production for wastewater treatment facilities.

3.2.2. PHB Production Using Mixed Photosynthetic Consortia

Another approach used for PHB production is the feast-famine strategy, which uses a mixed consortium of algae and cyanobacteria [85–88]. During this regime, the feast operation consists of a mixed culture of cyanobacterial consortium cultivated in a sequencing batch reactor (SBR) without aeration using acetate as a carbon source and light as an energy source [86]. During the famine phase, the NADH or the NADPH reserves of the cell is consumed using the oxygen produced by the algae cells present in the consortia leading to accumulation of around 20% (DCW) PHA [89]. Furthermore, maximum polymer content of 60% (DCW) of PHA was produced by a photosynthetic mixed culture in a permanent feast regime using high light intensity [86]. The anaerobic dark energy generation's capability of cyanobacteria is already been known [90]. Some cyanobacteria have also been known for their fermentation capability at the expense of their carbohydrate reserves [91]. The axenic dark feast conditions facilitated the acetate uptake, increasing the productivity significantly (up to 60%) (DCW), as the famine phase was eliminated [85,86]. The anaerobic fermentation of cyanobacteria to produce PHB has a potential, while the need for sterilization and aeration is eliminated, reducing also the energy costs. However, the source and cost of the substrate used remains a cost driver issue.

3.2.3. PHB Production Using Mixed Feed Systems

Production of PHAs in cyanobacteria can occur during phototrophic growth, using CO_2 as a sole carbon source and light energy, and also during heterotrophy, when using sugar supplementation. It has been estimated that the carbon substrate in a large-scale manufacturing context would constitute approximately 37% of the total production costs [27,92]. However, in order to cope with the low phototrophic PHB productivity in cyanobacteria, various studies have used supplementation of other carbon sources. The mini-review by Singh and Mallick has summarized the wild-type and recombinant cyanobacterial strains, their PHA content, and the substrate used for the biosynthesis of the biopolymer [24]. However, in most reported cases [34,46,93–97] of heterotrophic PHB production, the biomass concentrations produced are less than 1 g L^{-1} and increases in volumetric productivities are not described. Even though the PHB productivity increases in terms of biopolymer content (%DCW) using mixed feed systems, the use of external carbon substrates increases the production costs and also raises the question of the economic feasibility of PHB production from cyanobacteria. As long as heterotrophic organisms produce PHB at much higher rates than cyanobacteria, the only sense for commercialization of cyanobacterial PHB would be the sustainability. Therefore, research must focus on improving the phototrophic PHB production with the aim of increasing CO_2 uptake rates of cyanobacteria, with the support of viable bioprocess technology tools.

3.2.4. CO_2 Sequestration

CO_2 is a major greenhouse gas; its emission into the atmosphere has gradually increased in the past decades, causing global warming and its associated problems [98]. Carbon contributes to all organic compounds and is the main constituent of cyanobacterial and all biomass, amounting to up to 65% of DCW [79]. The industrial production of PHB that uses CO_2 feedstocks helps reduce the environmental impacts of CO_2 emission. Various studies have shown that an increase in CO_2 concentration during a cyanobacterial cultivation may increase the production of carbon reserve compounds, such as PHB. Markou et al. showed that an increase in carbon content leads to the production of carbon reserve compounds, such as lipids and PHAs [79]. The increase in the concentration of the carbon source also increased biopolymer accumulation in cyanobacterial strain *Spirulina* sp. LEB 18 [77]. However, what has not been discussed in the literature thus far are the effects of the day-night cycle on the CO_2 uptake rate and the productivity of carbon reserve compounds in cyanobacteria. Since CO_2 fixation occurs during the light phase, the total productivity and CO_2 sequestration rate will be lower in outdoor cultivations. During the dark phase, CO_2 utilization is minimized and the productivities are lowered and some carbon reserve molecules, such as glycogen, degrade. Other methods would need to be used to temporarily sequester CO_2 as a carbonate species during nighttime, which could then be utilized by cyanobacteria when the light is available again [99].

4. Production Strategies

The economic efficiency of any production process is indicated by the productivity, which comprises of growth rates, specific production rates, and the biomass concentration of the culture. Therefore, the economic efficiency of the production process will increase only when the mentioned parameters are improved. Once the strain and cultivation parameters are selected and optimized for the production process, the process performance can be considered and analyzed.

4.1. Cultivation Modes

Cyanobacteria producing PHA have been classified into two groups based on the culture conditions required for efficient polymer synthesis: group one requires a limitation of an essential media component for PHA synthesis; the second has no requirement for nutrient limitation for the accumulation of the polymer [100]. For industrial production of PHB, the second group is favorable for growth as it is accompanied by polymer synthesis.

In general, cultivation of cyanobacteria for the production of PHB can be done using various cultivation modes. The most common approach is using batch cultivation, in which the production of PHB is induced by a limiting nutrient or, in an ideal case, the production of the polymer becomes growth dependent. For the batch cultivation with the group one cyanobacteria, the concentration of nitrogen and phosphorus in the media play the key-role facilitating biomass growth, and their limitation trigger PHB synthesis. Thus, in such a process the cell growth is maintained without nutrient limitation, until the desired concentration is reached. Then, an essential limitation allows for efficient polymer accumulation. So far, a few studies have focused on optimizing the nutrients for the batch production of PHB in large-scale; others mainly have been done in flasks. Batch cultivation of *Synechocystis* sp. PCC 6803 using a nitrogen concentration of half of the optimal BG-11 media, showed 180 mg L^{-1} PHB from CO_2 [74]. The maximum PHB content of 125 mg L^{-1} was obtained for the non-sterile batch cultivation of *Synechocystis* sp. CCALA192 in a 200-L tubular photobioreactor [42]. Moreover, PHB was produced using optimized media in a one-liter tank reactor for the wild-type cyanobacterial strain *Synechocystis* sp. PCC 6714 obtained 640 mg L^{-1} of polymer [62].

The other common strategy for the cyanobacterial PHB production is using the SBR mode of operation, where growth and production occur in different reactors. In the growth vessel, media components are provided in abundance to facilitate maximum biomass production. In the induction photobioreactor, one or more media components are limited facilitating PHB biosynthesis. During the induction, residual biomass concentration remains more or less constant, while cell concentration

increases only by intracellular polymeric accumulation [101]. In order to facilitate higher productivities, both reactors can operate as chemostats. Thus far, no reports of cyanobacterial PHB production in SBR or chemostat mode have been described.

4.2. Cultivation Systems

Another challenge in commercial cyanobacterial production is associated with biomass production. There are three main production systems used for large-scale cultivation of microalgae and cyanobacteria. The most basic approach for the cultivation of photosynthetic organisms is the use of large natural locations, which is mostly done for microalgae such as *Dunaliella*. Releasing into natural locations is regarded as a deliberate release into the environment, since there are no effective protective measures to prevent the microalgae from entering the surroundings [60]. The other approach is the use of open raceway pond systems, which has been commonly applied worldwide. When these raceway ponds are used outdoors, the cultivation are regarded as a deliberate release, so the spread of genetically modified organisms cannot be excluded in this case [60]. Even though these systems are economically feasible, the maintenance of monocultures and improving productivity are the main bottlenecks associated with such cultivations. The use of an open pond system has so far been reported in a wastewater treatment facility, containing fish pond discharge that uses the cyanobacterial strain *Aulosira fertilissima*, which shows a PHB productivity of up to 92 g m^{-3} [80]. The third system is the sophisticated, closed production system: photobioreactors (PBR). These systems can be both placed in greenhouses to obtain more defined cultivation conditions or be installed outdoors. PBR systems are more flexible for the needs of the cultivation process and the desired species. The industrial-scale production of photoautotrophic cyanobacterial PHB has not been widely reported in photobioreactors. The various photobioreactor systems used to cultivate cyanobacteria is given by Koller et al. [102]. Yet Troschl et al. has described the cultivation of *Synechocystis salina* CCALA192 in a 200-L tubular photobioreactor for the production of PHB from CO_2 [23]. The maximum PHB productivity obtained under nitrogen limitation was 6.6% (DCW), while the volumetric and specific productivities were not reported in this case. Moreover, a mixed consortium of wastewater born cyanobacteria was cultivated in a 30-L PBR, showing a maximum productivity of 104 mg L^{-1} under phosphorus limitation [103].

5. Process Monitoring and Control

Today the most commonly used method for accurate determination of PHAs in bacterial cultivations is gas chromatography (GC) [104] or high-performance liquid chromatography (HPLC) [105,106]. These methods involve hydrolysis, subsequent methanolysis, or propanolysis of the PHAs in whole cells, in the presence of sulfuric acid and chloroform [107]. These extraction methods are laborious, time-consuming, and the optimum time of harvest might be lost due to the time needed for the analysis. Other methods for PHA analysis include gravimetric, infrared spectroscopy of chemically extracted PHB, fluorimetry, and cell carbon analysis [107–109]. It is necessary to develop viable analytics to help the development of an efficient commercial production process that enables monitoring and control of production, along with a rapid feedback on the state of the process.

Fourier transform infrared (FTIR) spectroscopy has been applied to determine the chemical composition of cyanobacteria with major cellular analytes, such as proteins, lipids, polysaccharide, nucleic acids, and PHAs [107,110]. It has been shown that FTIR spectroscopy can monitor water-soluble extracellular analytes in fermentation systems, as well as being an indirect method to determine the stage of fermentation by monitoring the physiological state of the cells [111]. Various studies have shown the potential of FTIR spectroscopy for determination of intracellular PHA contents in various microorganisms [107,112]. In the same direction, Jarute et al. have introduced an automated approach for on-line monitoring of the intracellular PHB in a process with recombinant *E.coli*, which uses stopped-flow attenuated total reflection FTIR spectroscopy [113]. In the case of cyanobacteria, there exists no such studies reporting on-line or at-line determination of intracellular carbon compounds, such as PHAs and glycogen. The measurements used and the parameters controlled in microalgae processes are specific in-line probes, such as pO_2, pCO_2, pH, and

temperature. In cyanobacterial industrial processes, spectroscopic measurement techniques, such as FTIR, can be used for monitoring, controlling the production, and determining the time of harvest. The on-line determination can also identify the limitation time and the limiting components based on the cell physiology, thus helping to make the cyanobacterial PHA production robust and manageable.

6. Production Scenarios

In order to compete with synthetic and other starch-based polymers in the market, the cost of cyanobacterial PHB needs to be reduced significantly. Yet, no economic analysis has been done to estimate the production costs of phototrophic PHB production. It has been reported that the cost of PHB production from heterotrophic organisms is in the range of 2–5 € kg^{-1} [114]. This value is still much higher than the estimated cost of petrochemical-based mass polymers like PE, PP, or PET, which is around 1.2 € kg^{-1} [114] and less. Taking into account the much lower time space yield and the biomass productivity in cyanobacteria and complications associated with the downstream processing, the cost associated with the production of PHB in cyanobacteria could be higher than that of heterotrophic microorganisms (>5 € kg^{-1}). Typically, more than 4.3 kg of sugar is needed to produce 1 kg of PHB [85]. Nevertheless, higher yields of product per substrate consumed have also been reported, showing values of 3.1 kg sucrose/kg PHB and of 3.33 kg glucose/kg of the polymer [15,115]. In this context, the substrate costs can be avoided by photoautotrophically produced PHB by cyanobacteria. However, the lower productivities of cyanobacteria will still increase the costs significantly. Among the main factors contributing to the cost of PHA production are equipment-related costs, such as direct-fixed-capital-dependent items, overheads, and some labor-dependent factors, which considerably increase with a decrease in productivity [27,101]. Therefore, for the production of the same amount of PHA per year, the process with lower productivity requires larger equipment [27,116]. To that end, one approach could be to reduce the costs associated with the building of photobioreactors. This can be accomplished by simplifying the design and the material used for the production of photobioreactors and their energy consumption [22]. Another alternative to increase the size of the facility or reactor while also reducing production costs is to use open pond raceways and wastewater born cyanobacteria instead of fresh water strains. However, it should be taken into account that the increase in volume will directly increase the effort associated with the downstream processing [25].

Moreover, it has been shown that using industrial flue gases may reduce the production cost of cyanobacterial biomass to around 2.5 € kg^{-1}, while using wastewater can decrease the costs further, to less than 2 € kg^{-1} [22,117]. Therefore, as already discussed, wastewater streams with high carbon, nitrogen, and phosphorus that are mix-fed with CO_2 from industrial flue gases, can be used to make the PHB production from cyanobacteria more efficient. Furthermore, producing several chemicals from the same microalgae feedstock could potentially make the production of multiple commodity chemicals from a biological resource economically viable [92].

7. The Remaining Challenges in Photosynthetic PHB Production

Current industrial PHA production processes rely mostly on the availability of agricultural resources, which are unsustainable (compare the food versus fuel discussion with first-generation biofuels) and leave a large ecological footprint [65].

In the case of cyanobacterial, PHA production research has mainly focused on genetic engineering to increase productivity, which mainly reports as higher % DCW polymer content. The studies have rarely reported an increase in photosynthetic efficiency or an increase in the specific growth rates and production rates. So far, very few studies have shown the use of wastewater-open pond systems for the production of PHAs. For a recent review of PHA production, see Koller et al. [118].

8. Outlook

Currently, the global research efforts directed towards individual aspects of cyanobacterial PHA production mainly focus on improved strains and recovery processes. Although various challenges are associated with the efficiency of the cyanobacterial PHA productivity and the extraction and purification of PHAs, optimization of each step separately will waste considerable effort and result in overall sub-optimality [27]. With respect to commercialization and scale-up of the cyanobacterial PHA production, the view of the whole processes needs to be considered. More attention towards sustainable and viable upstream processing may help to reach an economic PHA production point. Cyanobacterial PHA production, from an economic point of view, will only make sense if a continuous process can be achieved, especially using waste streams as a carbon source and for the media. The process can then be coupled with the bioremediation of agricultural and industrial effluents. Thus far, some wild-type and improved cyanobacterial strains are reported with PHB content which is mostly cultivated under controlled, defined, and sterile lab conditions. For production in industrial scale that is done under unsterile conditions, only *Nostoc moscorum* as an example is reported. Other strains are not tested or can hardly tolerate the harsh outdoor conditions. Although we have emphasized the importance of optimized media and cultivation conditions on PHA productivity, sustainable and viable commercial processes conducted under unsterile conditions using waste streams and open systems are required. Research needs to focus on screening for more robust strains, such as wastewater born mixed-cultures that can tolerate fluctuations in cultivation conditions like pH, temperature, salinity, and media composition. Furthermore, the durable strains for which production of PHB is associated with biomass growth and therefore the time-spaced yield will be improved.

PHA shows both the advantages of biobased carbon content and full biodegradability. In addition, cyanobacterial PHA can be more sustainable and more cost effective in the marine environment and when compared to PHA from carbohydrate fermentation. It can be a carbon-negative material, making the process not only attractive for PHA converters and users, but also for CO_2 emitters, like power stations. There are plenty of medium-size CO_2 point sources, e.g., biogas production facilities, where the CO_2 could be used in an adjacent cyanobacterial PHA factory erected on the non-arable land. Preferably, a biorefinery approach would be executed, where valuable compounds such as phytohormones and pigments are extracted from the cyanobacteria; then. PHA and biomass is anaerobically digested in a biogas plant, yielding a cost-effective and fully integrated process.

It is expected that over the next two decades there will be a shift toward more recycling of fossil-based and conventional plastics, with an accompanying reduction in material variety to facilitate collection, processing, and reuse. Moreover, we will see a maturing of the bioplastics industry, with more applications being developed with bioplastics, other than "gimmick" giveaways and small household and kitchen tools. Due to the unique and interesting property set of PHA, it can be anticipated that these materials, particularly PHB and its copolymers such as PHBV, will gain significance.

Author Contributions: D.K. and C.H. designed this study; D.K. carried out the research and wrote this manuscript, with the support of M.L. and C.H; the study was initiated by M.L. and C.H.; C.H. supervised this project; D.K., M.L., and C.H. have several years of practical research experience with PHB production from cyanobacteria focused on strain improvement, bioprocess optimization, and scale-up.

Funding: This research was funded by the authors' institutions.

Acknowledgments: The authors would like to thank the Vienna Business Agency for supporting the funding of this work.

Conflicts of Interest: The authors declare no conflict of interest.

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5.2 Cyanobacteria as production platform; strain screening and improvement

5.2.1 Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714

Abstract: Poly-β-hydroxybutyrate (PHB) production from CO_2 makes it possible to reduce the production cost of biodegradable polyesters. In this study unicellular cyanobacterium, *Synechocystis* sp. PCC 6714 has been identified as an unexplored potential source for production of PHB. *Synechocystis* sp. PCC 6714 was studied under various cultivation conditions and nutritional limitations. Combined effects of nitrogen and phosphorous deficiency lead to highest PHB accumulation under photoautotrophic conditions. Multivariate experimental design identified the optimal cultivation conditions for PHB accumulation. Biomass growth and PHB accumulation were studied under controlled defined conditions in a lab-scale photobioreactor. Rate of biomass growth was 4-fold higher in bioreactor experiments when cultivation conditions were controlled. After 14 days of cultivation in N and P, limited media intracellular PHB levels reached up to 16.4 % from CO_2. Scanning electron microscopy of *Synechocystis* sp. PCC 6714 showed many electron transparent PHB granules of N and P limited cells with an average diameter of 0.8 μm.

This manuscript is published in the Journal of Applied Microbiology and Biotechnology Express.

Kamravamanesh Donya, Pflügl Stefan, Nischkaur Winfried, Limbeck Andreas, Lackner Maximilian, Herwig Christoph. Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714. Journal of applied microbiology and biotechnology express. 2017; 7:143.

Kamravamanesh *et al. AMB Expr (2017) 7:143*
DOI 10.1186/s13568-017-0443-9

AMB Express

ORIGINAL ARTICLE Open Access

Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714

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Abstract

Poly-β-hydroxybutyrate (PHB) production from CO_2 has the potential to reduce the production cost of this biodegradable polyesters, and also to make the material more sustainable compared to utilization of sugar feedstocks. In this study the unicellular cyanobacterium, *Synechocystis* sp. PCC 6714 has been identified as an unexplored potential organism for production of PHB. *Synechocystis* sp. PCC 6714 was studied under various cultivation conditions and nutritional limitations. Combined effects of nitrogen and phosphorus deficiency led to highest PHB accumulation under photoautotrophic conditions. Multivariate experimental design and quantitative bioprocess development methodologies were used to identify the key cultivation parameters for PHB accumulation. Biomass growth and PHB accumulation were studied under controlled defined conditions in a lab-scale photobioreactor. Specific growth rates were fourfold higher in photobioreactor experiments when cultivation conditions were controlled. After 14 days of cultivation in nitrogen and phosphorus, limited media intracellular PHB levels reached up to 16.4% from CO_2. The highest volumetric production rate of PHB was 59 ± 6 mg L^{-1} day^{-1}. Scanning electron microscopy of isolated PHB granules of *Synechocystis* sp. PCC 6714 cultivated under nitrogen and phosphorus limitations showed an average diameter of 0.7 µm. The results of this study might contribute towards a better understanding of photoautotrophic PHB production from cyanobacteria.

Keywords: Photobioreactor cultivations, Cyanobacterium, Poly-β-hydroxybutyrate, *Synechocystis* sp. PCC 6714, Nitrogen and phosphorus limitation

Introduction

Today, petroleum-based plastics are an essential part across all industries and have replaced glass and paper in packaging (Khanna and Srivastava 2005). Global plastics production reached around 322 million tonnes in 2015 (Plastics Europe 2016). Accumulation of these non-biodegradable plastics in the environment is a worldwide concern (Thompson et al. 2009), e.g. as microplastics in the marine environment. In this context, attention has been focused on research for the production of biodegradable plastics (Samantaray and Mallick 2015). Polyhydroxybutyrate (PHB) is the best-characterized member of the polyhydroxyalkanoate (PHA) family and is widespread in various bacterial species as storage material (Liebergesell et al. 1994; Steinbüchel 2008).

PHB is biodegradable (and compostable according to EN 13432), insoluble in water, non-toxic and biocompatible. Therefore, PHB could be an attractive alternative to petroleum-based plastics (Samantaray and Mallick 2015). It resembles the commodity polymer polypropylene in its properties (Lackner 2015). PHB is commercially produced by heterotrophic bacteria such as *Alcaligenes eutrophus* (Madison and Huisman 1999), *Alcaligenes latus* (Grothe and Chisti 2000) and recombinant *Escherichia coli* (Schubert et al. 1988). Despite relatively high yields of PHB, production from bacterial fermentation requires sugar supplementation and continuous oxygen

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supply which results in high substrate and operation costs (Steinbüchel 2008; Wu et al. 2001). In addition, public discussion about bioplastics production from sugar feedstocks is similar to the discussion about first generation biofuels. Competition of material with food and feed production for the same resources potentially leads to shortages and price increases causing poverty and is also contributing to climate change through direct and indirect land use change (Chen et al. 2017).

In order to compete with common plastics, cost reduction in PHB production is obligatory. This could possibly be achieved using cheap substrates such as whey, hemicellulose, and molasses (Alias and Tan 2005; Reddy et al. 2003). In this context, cyanobacteria are emerging as an alternative host system due to their minimal nutrient requirements and photoautotrophic nature (Samantaray and Mallick 2012). To date, PHB accumulation has been reported for a few cyanobacterial species with photoautotrophic PHB production reaching less than 10% of dry cell weight (dcw) (Bhati et al. 2010). The thermophilic cyanobacterial strain *Synechocystis* sp. MA-19 is the sole exception with accumulation of 27% (dcw) PHB reported (Miyake et al. 2000). A higher PHB content in cyanobacteria has only been detected in the case of heterotrophy or genetic modification of the strain; 29 and 41.6% (dcw) PHB were obtained in the presence of acetate and under P-deficiency in *Synechocystis* sp. PCC 6803 and *Nostoc muscorum*, respectively (Panda et al. 2006; Sharma et al. 2007).

The aim of this study was to quantitatively investigate the unicellular non-nitrogen fixing cyanobacterial strain *Synechocystis* sp. PCC 6714 for its ability to produce PHB. This strain has not been studied yet for PHB production but its recently published genome sequence (Kopf et al. 2014) showed a close relation to the widely studied model organism *Synechocystis* sp. PCC 6803 which has been extensively studied for PHB accumulation. Hence, the potential of PHB accumulation in *Synechocystis* sp. PCC 6714 was evaluated by shake flask experiments as well as bioreactor cultivations under defined conditions and the volumetric rates and productivities were evaluated. Additionally, growth behavior on different carbon sources as well as different cultivation conditions and their effect on biomass and PHB formation were investigated. Finally, the morphology of PHB granules has been studied using scanning electron microscopy (SEM). Advanced quantitative process development approaches including multivariate experimental design were used, aiming to provide a quantitative and consistent data-set to the scientific community.

Materials and methods

Strain and growth conditions

An axenic culture of wild-type strain *Synechocystis* sp. PCC 6714 was purchased from Pasteur Culture Collection of Cyanobacteria (Pasteur Institute, Paris, France). Unless stated otherwise, *Synechocystis* sp. PCC 6714 was grown in BG-11 medium (Rippka et al. 1979) supplemented with 10 mM HEPES buffer pH 8.5 and 5 mM $NaHCO_3$ as carbon source prior to inoculation. In order to induce nitrogen deficiency, cells were cultured in BG-11 media without nitrate and ammonia. Ferrous ammonium citrate and Co $(NO_3)_2{\cdot}6H_2O$ were substituted with equimolar concentrations of Ferric citrate and $CoCl_2{\cdot}6H_2O$ in terms of iron and copper content. For phosphorus limitation, KH_2PO_4 was replaced with an equimolar concentration of KCl in terms of potassium content (Panda et al. 2006).

The impact of acetate supplementation on PHB accumulation was studied by taking cells from their late exponential growth phase and transferring them into media without nitrogen or without nitrogen and phosphorus source and 5 mM acetate as substrate. PHB content was analyzed on 3rd, 7th, 14th and 16th day of incubation.

Cultivations were done in 500 mL Erlenmeyer flasks containing 100 mL medium at 28 ± 2 °C under continuous illumination with 50 ± 5 µmol photon m^{-2} s^{-1} in photosynthetically active radiation (PAR) in a shaking incubator (Infors, Switzerland) at 100 rpm agitation.

The impact of different carbon sources on biomass growth was studied in shake flask experiments under continuous illumination using BG-11 media and 5 mM of either carbonate, acetate, glucose or glycerol.

Growth determination and estimation of dry weight

Biomass growth was determined spectrophotometrically at 750 nm using a UV–Vis Spectrophotometer (Thermo Scientific, USA) at 24-h intervals. Dry Cell Weight (dcw) was determined in triplicates by transferring 10 mL of fermentation broth or shake flask cultures to a reusable pressure filter holder (Sartorius, Göttingen, Germany) and by filtering on a pre-weighed 0.45 µm Cellulose acetate filter paper (Sartorius, Göttingen, Germany) at a pressure of 6 bars for 1 min. Filters were dried overnight at 60 °C and dry weight was determined gravimetrically. A correlation between optical density and dcw could be established and described in Eq. (1)

$$C_x = 0.425\ OD_{750}. \quad (1)$$

Gas-exchange limitation or heterotrophy and nutrient limitations

Impact of gas exchange limitation (i.e. limitation of gas transfer to the culture vessel) was studied using cultivations on media without nitrogen or without nitrogen and phosphorus source in 150 mL anaerobic flasks plugged with rubber stoppers under three different illumination conditions, dark, light and dark/light cycle (16:8) h, respectively.

Design of experiments

Full factorial experimental design with three center points and data evaluation were carried out using MODDE (Umetrics, Sweden). For each selected response, MODDE generates a multiple linear regression (MLR) model with key parameters of R^2 (coefficient of determination), Q^2 (predictability), RP (reproducibility) and MV (model validity). The thresholds for R^2, Q^2, and RP were 0.5 for each parameter. Models with an MV value of >0.25 were considered to be significant.

Bioreactor cultivations

Bioreactor experiments were carried out under sterile conditions in a 1.5 L jacketed glass reactor with a working volume of 1 L (Applikon B.V, the Netherlands). The temperature was maintained at 28 °C and pH was measured with a pH-electrode (Mettler Toledo GmbH, Vienna, Austria) and was automatically maintained at 8.5 by the addition of 0.5 M HCl or 0.5 M NaOH. Agitation speed was at 300 rpm. Gas flow was controlled by mass flow controllers for air and CO_2 (Brooks Instrument, Matfiels, USA). The reactor was bubbled with a mixture of sterile filtered air and 2% CO_2 at a flow rate of 0.02 vvm (20 mL min^{-1}). The illumination was done using LED strips wrapped around the reactor vessel providing a light intensity of 40 µmol m^{-2} s^{-1} photons in PAR. O_2 and CO_2 were measured in the off-gas with BlueSens gas sensors (BlueSens GmbH, Herten, Germany).

All fermentation parameters and variable pump setpoints were controlled using the process information management system Lucullus online monitoring system 3.2 (Securecell AG, Schlieren, Switzerland).

Photobioreactor experiments were performed in duplicates and one is shown as an example. Samples were taken in triplicates at 24-h intervals and were analyzed for biomass, PHB content, and concentrations of macronutrients.

Determination of the PHB content

PHB quantification was done using the procedure described by Schlebusch and Forchhammer (2010) and Taroncher-Oldenburg et al. (2000). Pre-weighed dried cells (5–10 mg) were boiled with 1 mL conc. H_2SO_4 at 100 °C on a heating block (Accublock™, Labnet, USA) for one hour to convert PHB to crotonic acid. Samples were allowed to cool down and subsequently diluted 20 times using 0.014 M H_2SO_4. Crotonic acid was determined using a high-performance liquid chromatography system (Thermo-Fischer Scientific, USA) with a Nucleosil C8 column (Macherey–Nagel, Germany) using an isocratic method. The mobile phase used was 20 mM NaH_2PO_4 buffer; pH 2.5 and acetonitrile (70:30) with a flow rate of 0.85 mL min^{-1} and a column temperature of 30 °C. Detection of crotonic acid was done using a diode array detector (DAD) detector (Thermo-Fischer Scientific, USA) at 210 nm. For calibration, pure PHB (Sigma-Aldrich, USA) was treated accordingly and analyzed in parallel with samples. Instrument control and peak evaluation were done with Chromeleon 7.2 (Thermo-Fischer Scientific, USA). The percentage (dcw) PHB was determined with the amount of PHB obtained from HPLC analysis and the cell dry weight of biomass used for the analysis using Eq. (2):

$$\%\ \text{dcw}\ PHB = \frac{mg(\text{PHB})}{mg(dcw)} * 100. \quad (2)$$

Analysis of micronutrients

The concentrations of nitrogen (N), phosphorus (P), sulfur (S), and carbon (C) were measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES) according to the modified method described previously by Nischkauer et al. (2014). For the analysis, samples were diluted (1:2) with 1% (v/v) hydrochloric acid and iridium was added as internal standard (final concentration 5 µg mL^{-1}). Calibration solutions were prepared from the analytical grade salts of (sodium dihydrogen phosphate dihydrate for P, potassium nitrate for N, sodium sulfate for S, and sodium acetate for C). Samples and standards were analyzed with an iCAP 6000 ICP-OES instrument (Thermo Scientific, Germany). The optimized ICP parameters are given in Table 1. For each element three replicates were measured, two emission lines were monitored (for nitrogen, only one suitable line was available in the investigated spectral range), and quantitative results were calculated from both emission lines. The counts observed were converted into concentration units by means of external aqueous calibration. The response of the internal standard (Ir) was constant over each measurement session (5% relative standard deviation, no temporal trend),

Table 1 Represents the optimized ICP parameters used for determination of nitrogen, phosphorus, sulfur and carbon

Exposure time	7 s
RF power	1400 W
Nebulizer gas flow	0.8 L min^{-1} argon
Viewing height above load-coil	11 mm
Cooling gas flow	15 L min^{-1} argon
Auxiliary gas flow	2 L min^{-1} argon
N 174.272 nm	[a]
P 213.618 nm	P 178.284 nm
S 182.034 nm	S 180.731 nm
C 175.183 nm	C 193.091 nm

RF radio frequency

[a] N has only one useful emission line in the spectral range investigated

and no difference in Ir-response between samples and calibration standards was observed.

Analysis of PHB granules using scanning electron microscopy (SEM)

50 mL cyanobacterial cell suspension containing PHB granules was homogenized using Panda Plus homogenizer (GEA Group AG, Düsseldorf, Germany) for ten passages at 1500 bar pressure at room temperature. The homogenate was centrifuged at 10,000 rpm at 4 °C for 10 min. In order to remove pigments, the pellet containing PHB granules was washed with methanol. The cell pellet was subsequently harvested by centrifugation at 3000 rpm and resuspended in ultrapure water. 100 µL of an appropriate dilution of the suspension was transferred on a gold-sputtered (10–50 nm) polycarbonate filter (Millipore-Merck, Darmstadt, Germany) using reusable syringe filter holders with a diameter of 13 mm (Sartorius, Göttingen, Germany) and filtered using pressurized air. Gold-sputtered filters were washed with 200 µL of ultrapure water and fixed on a SEM sample holder using graphite adhesive tape and subsequently sputtered with gold to increase the contrast. SEM was performed using a QUANTA FEI SEM (Thermo Fisher, Waltham, MA, USA) with a secondary electron detector (SED). The acceleration voltage of the electron beam was set between 3 and 5 kV. The diameter of the PHB granules on SEM pictures was evaluated using the ImageJ plugin Fiji (Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin-Madison, WI, USA).

Results

Strain characterization

Impact of carbon sources on biomass formation

Initial characterization by shake flask experiments was done to gain a better understanding of cyanobacterial strain *Synechocystis* sp. PCC 6714. Different carbon sources, namely carbonate, acetate, glucose and glycerol were studied with respect to their impact on biomass growth.

As shown in Fig. 1 growth on carbonate reached a maximum biomass concentration of 1.4 ± 0.15 g L^{-1} after 14 days of incubation with an average specific growth rate ($\mu_{average}$) of 0.095 ± 0.01 day^{-1} and a maximum specific growth rate (μ_{max}) of 0.225 ± 0.02 day^{-1}. Growth on acetate reached a maximum biomass concentration of 1.3 g L^{-1} after 14 days of cultivation with a $\mu_{average}$ of 0.09 ± 0.01 day^{-1} and a μ_{max} of 0.2 ± 0.02 day^{-1}, which are slightly lower when compared to growth on carbonate. Growth on glucose occurred with a $\mu_{average}$ of 0.107 ± 0.01 day^{-1} and a μ_{max} of 0.28 ± 0.02 day^{-1} which is higher when compared to growth on carbonate and acetate. Growth on glycerol occurred with a lag phase of 250 h. After that, a $\mu_{average}$ of 0.09 ± 0.01 day^{-1} a biomass concentration of 0.72 ± 0.1 g L^{-1} was observed. This value was comparable with cultivations on carbonate and acetate. The results showed that *Synechocystis* sp. PCC 6714 can grow under different conditions ranging from fully autotrophic to heterotrophic growth conditions.

PHB formation in Synechocystis sp. PCC 6714

In order to study whether *Synechocystis* sp. PCC 6714 is able to accumulate PHB, cultivations under various nutrient limitations and continuous illumination were performed. The impact of the nitrogen source during growth (nitrate or ammonia), gas exchange limitation (GEL) and heterotrophy were studied under nutrient limitation. Experiments were carried out in two steps. After the growth phase on complete BG-11 media using nitrate or ammonia as a nitrogen source, limiting conditions were achieved by transferring cells to media without a nitrogen or without a nitrogen and phosphorus source, while using carbonate or acetate as substrate. Table 2 shows the PHB content obtained for *Synechocystis* sp. PCC 6714 under

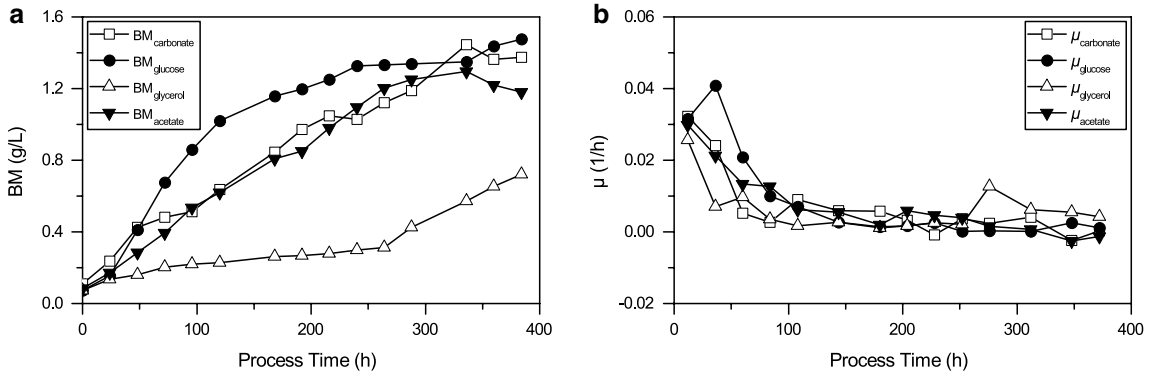

Fig. 1 **a** Growth curves and **b** average specific growth rates of *Synechocystis* sp. PCC 6714 on 5 mM carbonate, acetate, glucose, and glycerol (*BM* biomass). The values shown are the mean of three individual experiments, a single standard deviation is given as *error bar*

various limiting conditions. 7.5 ± 1% (dcw) PHB was obtained after 7 days of nitrogen limitation for cells previously grown on nitrate. This amount was further increased to 10 ± 1% (dcw) when cultivation was prolonged to 14 days. PHB content was higher when compared to cells previously grown on ammonia, where 9 ± 1% (dcw) were obtained after 14 days of limitation. After 16 days of nitrogen limitation in case biomass was grown on nitrate and acetate was the supplemented carbon source, 14.9 ± 1% (dcw) PHB was obtained. After 7 days of cultivation under nitrogen and phosphorus limitation, 8.2 ± 1% (dcw) PHB accumulation was detected and the highest PHB content of 13 ± 1% (dcw) was found after 14 days of limitation. The intracellular PHB level reached 15.5 ± 2% (dcw) after 16 days of incubation under nitrate and phosphate starvation when 5 mM acetate was supplemented. The PHB accumulation was higher for both nitrogen and nitrogen and phosphorus limitation when nitrate was used as nitrogen source. Gas exchange limitation under nitrogen and phosphorus limitation was done in order to study the effect of preventing an exchange of gas between the culture vessel and the environment on PHB accumulation. As shown in Table 2, a significant reduction in the PHB pool of *Synechocystis* sp. PCC 6714 was observed when the transfer of gas into the culture vessel was limited. The PHB content obtained was only 2.2 ± 1% (dcw) after 7 days of limitation and this amount was even further reduced when cultivation was prolonged to 14 days. Higher biomass concentrations were observed under nitrogen limitation than under nitrogen and phosphorus limitation. In addition, an insignificant increase in biomass concentration under GEL was observed.

Influence of cultivation conditions on PHB accumulation using a multivariate design of experiments (DoE)

In this study, the ability of *Synechocystis* sp. PCC 6714 to accumulate PHB was elucidated. The highest photoautotrophic PHB content of 13 ± 1% (dcw) was obtained under nitrogen and phosphorus limitation. To better understand the capability of the strain to produce biomass and PHB, the influence of temperature, pH, and CO_2 availability was investigated. To that end, a full factorial design of experiments under nitrogen and phosphorus limitations was carried out. The experiments and the parameters are given in Table 3. It was hypothesized that PHB accumulation in strain *Synechocystis* sp. PCC 6714 is influenced by cultivation conditions. The two responses, biomass growth, and PHB accumulation after 14 days were determined and the results of the DoE were analyzed with MODDE.

First, the effect of the different factors on biomass formation was determined. As shown in Fig. 2a high CO_2 concentration significantly reduced the biomass concentration. The model obtained showed that all three factors had a significant influence on the final biomass concentration. While higher CO_2 concentrations and temperatures decreased biomass formation, higher pH values

Table 3 Shows the individual experiments of the full factorial design of experiments for the screening study

Experiment no.	Temperature °C	pH	CO_2%
N1	25	7.0	2
N2	25	7.0	10
N3	25	10	2
N4	25	10	10
N5	35	7.0	2
N6	35	7.0	10
N7	35	10	2
N8	35	10	10
N9	30	8.5	6
N10	30	8.5	6
N11	30	8.5	6

Table 2 PHB concentrations in *Synechocystis* sp. PCC 6714 grown under continuous illumination and different limiting conditions

Limitation condition	3 day	7 day	14 day	16 day
Cells growing of complete BG-11 media	0	<0.5%	<1%	1%
Nitrogen limitation (growth on nitrate), %	2.5	7.5	10	9.5
Nitrogen limitation (growth on ammonia), %	2	6	9	8.5
Nitrogen limitation (growth on nitrate) and 5 mM acetate, %	3	7.9	11.4	14.9
Nitrogen limitation (growth on ammonia) and 5 mM acetate, %	3	7.2	11	14
Nitrogen and phosphorus limitation (growth on nitrate), %	3.4	8.2	13	11
Nitrogen and phosphorus limitation (growth on ammonia), %	2.9	7	12.5	12
Nitrogen and phosphorus limitation and 5 mM acetate, %	3.2	7	12.4	15.5
Nitrogen, phosphorus and gas exchange limitation, %	<1	2.2	1.5	1

The values shown are the mean of three individual experiments

favored biomass formation. Within the borders of the experimental matrix, highest biomass concentration of 1.12 ± 0.12 g L^{-1} was found at 2% CO_2, pH 10 and 25 °C.

Significant effects for the interaction terms CO_2 * pH (increased biomass), CO_2 * temperature (decreased biomass) and pH * temperature (decreased biomass) have also been obtained from the biomass model.

Next, the effect of the different factors on PHB accumulation was determined. As shown in Fig. 2c PHB accumulation was significantly reduced by increased CO_2 concentrations, pH values, and temperatures. However, the effect was not significant for the pH value. Within the borders of the experimental matrix, highest PHB accumulation of 103 ± 10 mg L^{-1} (11 ± 1% dcw) was found at 2% CO_2, pH 7 and 25 °C followed by an average PHB content of 10 ± 1% (dcw) at pH 8.5, 30 °C and 6% CO_2.

The DoE was done under three different illumination conditions: dark, light and dark/light cycle (16:8 h), respectively. The results obtained showed both dark/light cycle and continuous illumination were suitable for PHB accumulation. No significant difference was observed between the results obtained for maximum PHB accumulation under dark/light cycle (100 ± 10 mg L^{-1}, 10.5% dcw) and continuous illumination (103 ± 10 mg L^{-1}, 11% dcw). Dark/light cycle was found stimulatory for biomass growth. Maximum biomass concentration obtained under dark/light cycle (1.30 ± 0.1 g L^{-1}) was higher when compared to continuous illumination (1.12 ± 0.1 g L^{-1}). Cells grown without light showed very little biomass growth (< 0.1 g L^{-1}) and PHB accumulation (<1% dcw).

Photobioreactor cultivation of *Synechocystis* sp. PCC 6714

Based on the results of the design of experiments indicating favorable conditions for both biomass formation and PHB accumulation these two parameters were studied in photobioreactor cultivations. To that end, photoautotrophic cultivation of *Synechocystis* sp. PCC 6714 was established in a 1-L lab scale photobioreactor with the aim to determine whether defined cultivation conditions could improve biomass formation, specific growth rates, and PHB content. In addition, the impact of the nitrogen source on biomass growth was studied. The data from the design of experiments suggested different cultivation conditions for maximum biomass concentration and PHB accumulation under nitrogen and phosphorus limitations. To ensure suitable cultivation conditions for

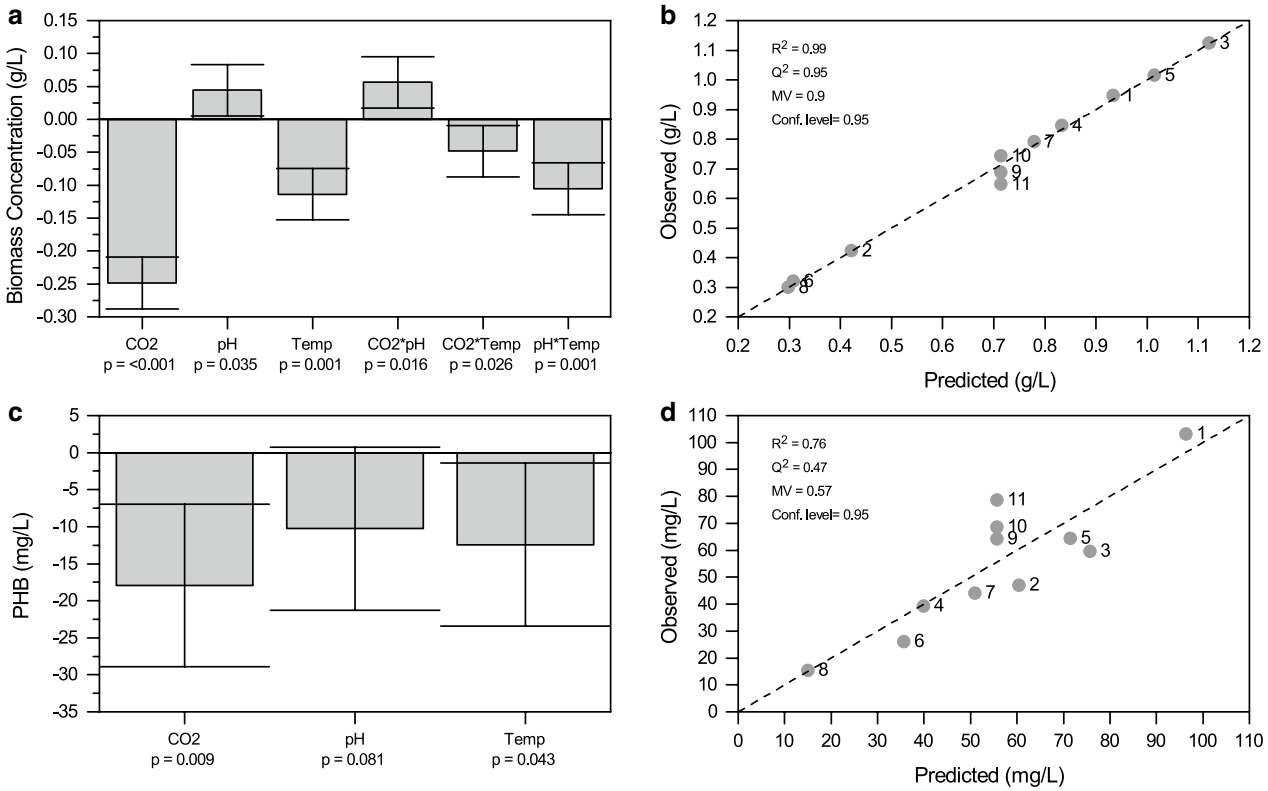

Fig. 2 **a**, **c** Represent the coefficient plots of the DoE experiments for *Synechocystis* sp. PCC 6714 under nitrogen and phosphorus limitation, for biomass concentration (**a**) and PHB accumulation (**c**), respectively. **b**, **d** Show the model parameters and observed-versus-predicted plots of the models obtained for biomass concentration (**b**) and PHB accumulation (**d**) under nitrogen and phosphorus limitation

biomass formation and PHB accumulation, four photobioreactor cultivations with different pH set points (7, 8.5, 9 and 10) at 28 °C and 2% CO_2 were done (data not shown). Highest biomass formation was observed at pH 8.5. Therefore, the following parameters of 2% CO_2, pH 8.5 and temperature of 28 °C were used for all photobioreactor cultivations.

Figure 3 shows growth curves obtained for cultivations a) in photobioreactor and b) in shake flasks with either nitrate or ammonia as nitrogen source. The $\mu_{average}$ in photobioreactor cultivations was 0.672 ± 0.07 day^{-1} with a μ_{max} of 0.792 ± 0.08 day^{-1} compared to $\mu_{average}$ of 0.168 ± 0.02 day^{-1} and μ_{max} of 0.240 ± 0.02 day^{-1} in shake flasks cultivations. This represents a fourfold higher specific growth rate for *Synechocystis* sp. PCC 6714 under defined conditions in the photobioreactor compared to shake flask cultivations.

The obtained results suggest that both nitrogen sources, nitrate and ammonia, are suitable for cultivation of *Synechocystis* sp. PCC 6714 in a photobioreactor and could facilitate biomass growth.

PHB accumulation in the photobioreactor cultivations

A significant increase of specific growth rate could be successfully shown in photobioreactor runs compared to shake flask experiments. Thus, it was hypothesized that also PHB accumulation is increased in photobioreactor cultivations. Two step cultivations of *Synechocystis* sp. PCC 6714 were done for photoautotrophic production of PHB under nutrient limitations.

As shown in Fig. 4a biomass growth occurred in BG-11 media using nitrate as nitrogen source until 120 h with a $\mu_{average}$ of 0.38 ± 0.04 day^{-1}. Limitation started when cells were harvested and transferred into media without nitrogen and phosphorus. Cell growth ceased after 200 h of cultivation although metabolic activity continued until the end of the process. PHB accumulation started at the early phase of the limitation and a PHB content of 8 ± 1% (dcw) after 6 days of limitation was obtained. PHB accumulated to an intracellular concentration of about 14 ± 1% (dcw) on day 9 and the highest PHB content was observed on day 15 of about 16.4 ± 2% (dcw). A decline in PHB content was detected after day 15. The maximum polymer content per volume of medium was 342 ± 30 mg L^{-1} PHB which corresponds to 14.6 ± 1% (dcw) on day 10 from CO_2. The highest volumetric PHB production rate obtained was 59 ± 6 mg L^{-1} day^{-1}.

Figure 4b shows biomass growth occurred on BG-11 media and ammonia as nitrogen source under a photoautotrophic condition in a lab-scale photobioreactor. Growth occurred until 120 h with a $\mu_{average}$ of 0.36 ± 0.04 day^{-1}. This value is lower when compared with cultivations with nitrate as a nitrogen source. The intracellular level of PHB at the starting point of the limitation phase was determined to be about 1% (dcw) which is higher than for the cultivation with nitrate. When ammonia was used as nitrogen source for growth, the maximum PHB content obtained under nitrogen and phosphorus limitation was 0.288 ± 0.3 mg L^{-1} PHB on day 19 which corresponds to 14 ± 1% (dcw) PHB. The highest volumetric PHB production rate with ammonia as nitrogen source was 51 ± 5 mg L^{-1} day^{-1}.

Elemental analysis of macronutrients from photobioreactor samples

The concentrations of macronutrients nitrogen, phosphorus, sulfur and carbon were characterized in photobioreactor cultivations of *Synechocystis* sp. PCC 6714

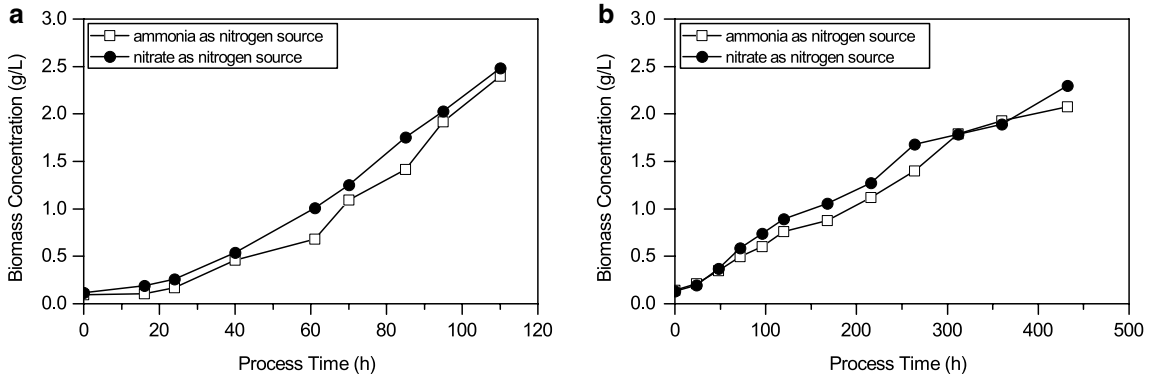

Fig. 3 Cultivation of *Synechocystis* sp. PCC 6714 on two different nitrogen sources, nitrate and ammonia, under continuous illumination. Given are biomass concentration over time for **a** photobioreactor cultivations and **b** shake flask experiments. The values shown are the mean of three individual experiments, a single standard deviation is given as *error bar*

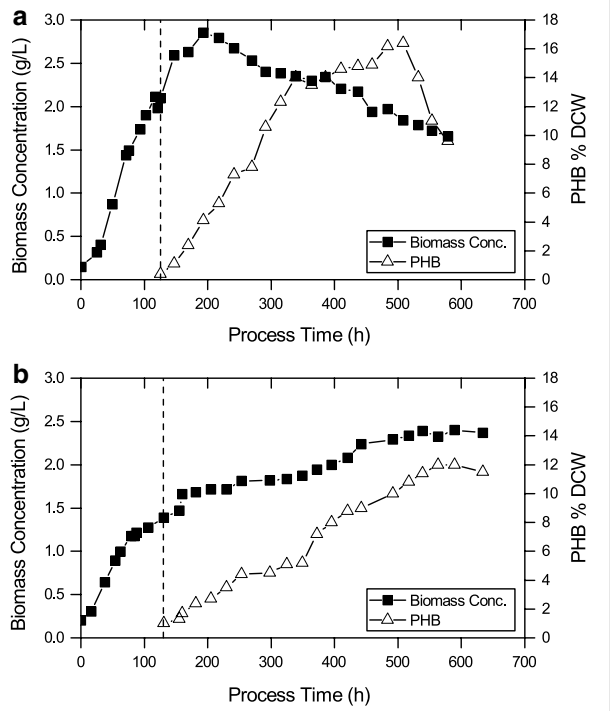

Fig. 4 Time course of growth and PHB accumulation under nitrogen and phosphorus limitation for photobioreactor cultivations of *Synechocystis* sp. PCC 6714 for **a** growth on nitrate **b** growth on ammonia. *The *vertical bar* represents the start of the limitation phase. The *values* shown are the mean of three individual experiments)

under photoautotrophic growth conditions with nitrate as the nitrogen source. Elemental analysis of the culture supernatant showed uptake of nitrogen and phosphorus during the first 50 h of the growth phase (0–120 h) (Fig. 5a). Nitrogen concentrations reduced to below the detection limit (70 mg L^{-1}) during the growth phase and remained below the detection limit until the end of the cultivation. Accumulation of carbon up to 6 ± 0.5 g L^{-1} in the culture supernatant was observed during both, the growth and limitation phase whereas no such accumulation of carbon was observed intracellularly. Uptake of small amounts of sulfur was detected both during growth and limitation phase. Figure 5b shows that the intracellular concentration of phosphorus increase during the first 25 h of the growth phase (0–120 h) and subsequently decreased until the limitation phase was started. During the limitation phase, the intracellular level of phosphorus remained almost constant. The intracellular concentration of carbon, nitrogen, and sulfur during both growth and limitation phase was below the detection limits.

Size determination of PHB granules

Apart from quantitative determination of the PHB content, it was of interest to visualize PHB granules in this strain. To this end, scanning electron microscopy (SEM) was used to determine shape and size of the PHB granules extracted from *Synechocystis* sp. PCC 6714. Figure 6 shows a SEM image of PHB granules from the photoautotrophic cultivation of nitrogen and phosphorus limited cells. Image analysis of PHB granule using ImageJ plugin Fiji showed electron transparent particles with average diameter of 0.7 µm and maximum size of 0.95 µm. Cells cultured in complete BG-11 media were used as a control. They were homogenized and prepared the same way for analysis by scanning electron microscopy. For the unlimited control cells, no electron transparent particles were observed.

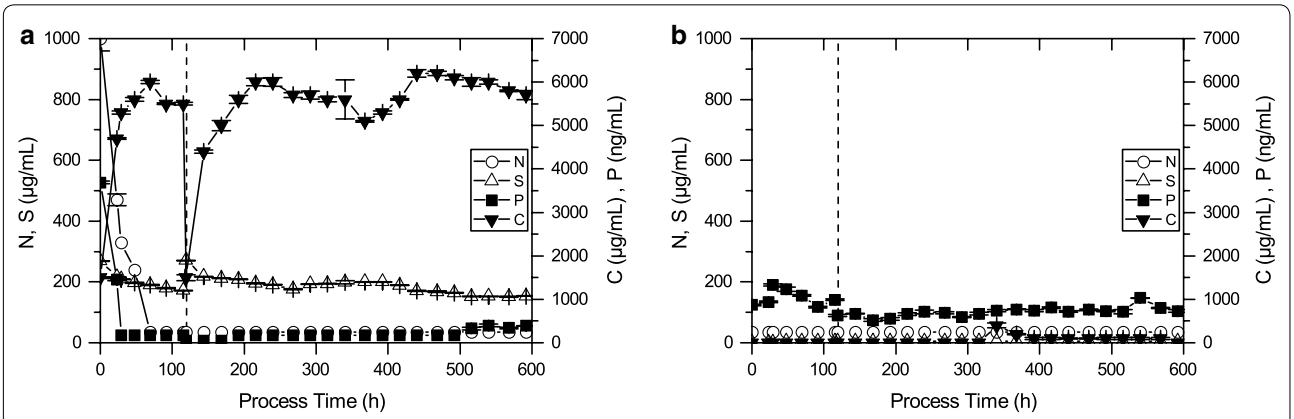

Fig. 5 Time course of residual macronutrient concentrations for nitrogen, phosphorus, sulfur, and carbon in photobioreactor cultivations of *Synechocystis* sp. PCC 6714 cultivated photoautotrophically and with nitrate as the nitrogen source is shown **a** in the culture supernatant **b** intracellular concentration of the macronutrients. *The *vertical bar* represents the start of the limitation phase. The *values* shown are the mean of three individual experiments, a single standard deviation is given as *error bar*

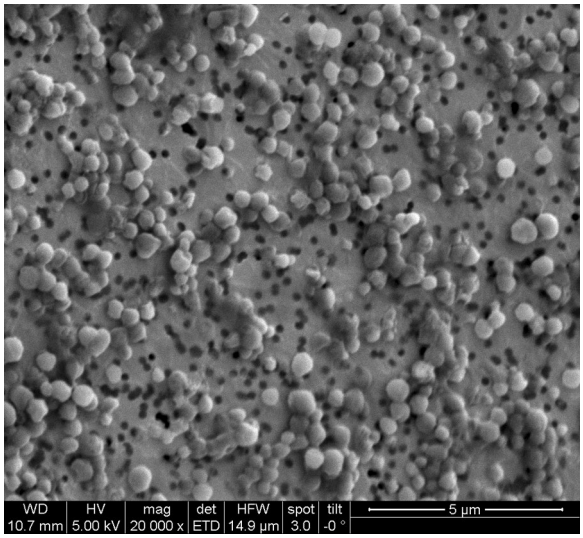

Fig. 6 Scanning electron microscopy image of PHB granules from *Synechocystis* sp. PCC 6714 cells cultivated photoautotrophically and N and P limitation

Discussion

Initial characterization

Initial strain characterization showed the flexibility of *Synechocystis* sp. PCC 6714 for utilization of different carbon sources for growth. Although growth on carbonate and acetate occurred with more or less the same $\mu_{average}$ 0.095 ± 0.01 day^{-1} for growth on carbonate (1.45 ± 0.1 g L^{-1}) higher biomass concentration was detected when compared to acetate (1.25 ± 0.1 g L^{-1}). This is in contrast to results which were shown for other cyanobacteria where acetate resulted in higher biomass concentrations (De Philippis et al. 1992). As it was expected, growth on glucose occurred at higher $\mu_{average}$ (0.107 ± 0.01 day^{-1}) and the observation was in line with previous reports of Vermaas (1996) and Wang et al. (2002) where higher growth rates were detected for *Synechocystis* sp. PCC 6803 when glucose was used as the substrate.

PHB formation under nutrient limitations

In this study, photoautotrophic PHB accumulation in *Synechocystis* sp. PCC 6714 has been explored for the first time. Under photoautotrophic conditions and nitrogen limitation, a rise in PHB pool up to 10 ± 1% (dcw) was observed. Stimulation of PHB accumulation by nitrogen limitation has been previously explored by Lee et al. (1995) and (2005) where an increase in PHB accumulation under nitrogen limitation was ascribed to high intracellular concentrations of NADPH. Supplementation of acetate under nitrogen deficiency was found to be stimulatory for PHB accumulation in *Synechocystis* sp. PCC 6714. This has also been observed for other cyanobacteria. Wu et al. (2002) reported 15 ± 1% (dcw) PHB accumulation for *Synechocystis* sp. PCC 6803 when acetate was supplemented. Increase in PHB production by acetate supplementation has been explained by Dawes (1992) as the result of direct utilization of the substrate for polymer synthesis. Simultaneous limitations of nitrogen and phosphorus under photoautotrophy appeared to be a suitable stimulant for PHB accumulation since up to 16.4 ± 2% (dcw) PHB were detected under these conditions. These results are in line with earlier findings of Pal et al. (1998) and Nishioka et al. (2001) where it was shown that PHB accumulation could be increased by phosphate unavailability for *Azotobacter chroococcum* and *Synechococcus* sp. MA-19, respectively.

Limitation of gas exchange under nitrogen and phosphorus deficiency in *Synechocystis* sp. PCC 6714 reduced the intracellular PHB pool and biomass concentration significantly. This could be due to an inhibitory effect of oxygen caused by a build-up in the culture vessel. Oxygen competes with CO_2 during photorespiration for ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme to convert CO_2 to biomass (Raso et al. 2012). The findings from this study are in contrast with the results reported by Panda and Mallick (2007) which showed GEL can significantly boost PHB content in *Synechocystis* sp. PCC 6803. The different results of the two studies could be due to the different conditions used for the experiments.

Optimization of cultivation parameters

Key parameters influencing growth and PHB content in *Synechocystis* sp. PCC 6714 were identified using a multivariate design of experiments. It was found that biomass formation and PHB accumulation were a function of various cultivation parameters. Within the borders of the specified experimental matrix, the highest biomass concentration (1.12 ± 0.1 g L^{-1}) and highest PHB accumulation (11 ± 1% dcw) were obtained at 2% CO_2 concentration. This observation, however, is in contrast to earlier findings of Eberly and Ely (2012) where growth rates and accumulation of carbon storage compounds were enhanced with increased CO_2 concentrations in the thermophilic cyanobacterium *Thermosynechococcus elongatus*. This difference in growth behavior might be due to lower CO_2 solubility at 50 °C used for *T. elongates* compared to 28 °C used for *Synechocystis* sp. PCC 6714. Additionally, a temperature of 28 °C used for photobioreactor cultivations of *Synechocystis* sp. PCC 6714 was favorable for PHB accumulation. This is in line with previous observations of Panda et al. (2006) for *Synechocystis* sp. PCC 6803 where the temperature range of 28–32 °C was preferred for PHB accumulation.

Cultivations with either continuous illumination or dark/light cycles did not show a significant difference in PHB content. However, cells grown without light showed very little biomass growth and PHB accumulation.

Cultivation of cyanobacteria in the photobioreactor

Interestingly, photobioreactor cultivations of *Synechocystis* sp. PCC 6714 showed fourfold higher average specific growth rate when compared to shake flask cultivations. This observation is likely due to well-defined conditions in the photobioreactor compared to uncontrolled shake flask cultivations. It has been shown that under controlled conditions, growth rates and biomass concentration can be improved in microalgae cultivations (García-Malea et al. 2009; Ugwu et al. 2008). This underlines the importance of defined conditions to obtain highly productive processes (Pruvost et al. 2011). It has been shown biomass and lipid productivity in microalgae can be improved significantly under defined conditions of a photobioreactor.

It was observed that for photobioreactor cultivations using ammonia as the nitrogen source during the growth phase, *Synechocystis* sp. PCC 6714 already showed a PHB content of 1% (dcw) at the end of the growth phase. This suggests that the cells might have already been limited during the growth phase. One explanation for this phenomenon might be that the uptake of ammonia is affected by the pH-dependent equilibrium between NH_3 and NH_4^+ and therefore might not be sufficiently available to the cells throughout the growth phase. Furthermore, it would be expected that uptake rates for ammonia are higher than for nitrate since ammonia can directly be utilized by the cell, whereas nitrate needs to be converted into ammonia first. Therefore, the energetic cost for the cell to take up ammonia is lower compared to nitrate (Dortch and Postel 1989; Syrett 1981). In the present study, higher growth rates and PHB contents were observed for cultivations with nitrate as nitrogen source compared to cultivations with ammonia as nitrogen source. This has been previously reported by Rückert and Giani (2004) where higher cell densities and a higher protein content was detected in *Microcystis viridis* when nitrate was used instead of ammonia or urea. In addition, high concentrations of ammonia have been shown to be toxic to some cyanobacteria (Drath et al. 2008), while no such toxicity has been reported for nitrate. Problems related to solubility and toxicity of ammonia could be overcome by implementing a continuous feeding strategy.

Potential of *Synechocystis* sp. PCC 6714 for photoautotrophic production of PHB

16.4% (dcw) PHB were obtained for *Synechocystis* sp. PCC 6714 from CO_2. PHB content of 11–13% and 29% (dcw) are reported, respectively, in wild-type and genetically modified *Synechocystis* sp. PCC 6803 by Klotz et al. (2016) and Khetkorn et al. (2016). Also 27 and 23% (dcw) for *Synechococcus* sp. MA-19 and *Nostoc muscorum*, respectively, have been obtained (Nishioka et al. 2001; Sharma and Mallick 2005). However, total product concentration and volumetric rates for these strains have not been described and therefore it is challenging to evaluate the mentioned strains in terms of PHB productivity only based on the reported percentage of dry cell weights. In this respect, Wu et al. in (2001) reported total PHB concentration of 16 to 27 mg L^{-1} PHB for *Synechocystis* sp. PCC 6803. For *Calothrix scytonemicola* TISTR 8095 PHB content of about 25.4% (dcw) was reported with a total PHB concentration of 356 mg L^{-1} after 60 days of cultivation (Kaewbai-Ngam et al. 2016). In this study, the maximum polymer content per volume of the medium of 342 ± 34 mg L^{-1} PHB was observed after 10 days of limitation which corresponds to 14.6 ± 1% (dcw) PHB. The highest volumetric production rate of PHB observed was 59 ± 6 mg L^{-1} day^{-1}. Available literature so far has not used this parameter, therefore our obtained value cannot be compared to other studies. Additional accumulation of a carbon-containing compound (up to 6 g L^{-1} of carbon) in the culture supernatant during photobioreactor cultivations of *Synechocystis* sp. PCC 6714 could be due to the formation of extracellular polysaccharides as previously reported by De Philippis and Vincenzini (1998). However, this observation requires further investigation.

In this study, the cyanobacterium *Synechocystis* sp. PCC 6714 has been reported as PHB producer for the first time. The results suggest *Synechocystis* sp. PCC 6714 to be suitable as a potential host strain for photoautotrophic PHB production. The specific growth rate, total PHB content and volumetric PHB production rate were increased significantly only by controlling process conditions in two-step batch cultivations in a photobioreactor. In order to obtain a robust process further optimization with respect to a suitable process strategy supporting both biomass formation as well as stable PHB production is required. A potential strategy that shall be further investigated is to optimize the media to provide nitrogen and phosphorus in a way that enables a one-step process without the need to exchange the media.

Abbreviations

PHB: polyhydroxybutyrate; dcw: dry cell weight; SEM: scanning electron microscopy; SED: scanning electron detector; $\mu_{average}$: average specific growth rate; μ_{max}: maximum specific growth rate; PAR: photosynthetically active radiation; OD_{750}: optical density at 750 nm; GEL: gas exchange limitation; DoE: design of experiment; MLR: multiple linear regression; MV: model validity; R^2: coefficient of determination; Q^2: predictability of a model; RP: reproducibility of a model; ICP-OES: inductively coupled plasma-optical spectroscopy; Ir: internal standard; RuBisCO: ribulose-1, 5-bisphosphate carboxylase/oxygenase.

Authors' contributions
DK, SP and CH planned the experiments, analyzed the data and wrote the manuscript. DK, WN and AL carried out the experiments, WN, AL and ML reviewed the manuscript. All authors read and approved the final manuscript.

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Acknowledgements
Open access funding was provided by TU Wien, Vienna, Austria. The authors would like to thank "Vienna Business Agency" for funding this project.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the main article.

Funding
This work was supported by the "Vienna Business Agency".

Publisher's Note

Received: 20 June 2017 Accepted: 22 June 2017
Published online: 06 July 2017

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5.2.2 Increased poly-β-hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: mutant generation and characterization

Abstract: Photosynthetic Poly-β-hydroxybutyrate (PHB) productivity in cyanobacteria needs to be increased to make cyanobacterial derived bioplastics economically feasible and competitive with petroleum-based plastics. In this study, high PHB yielding mutants of *Synechocystis* sp. PCC 6714 have been generated by random mutagenesis, using UV light as a mutagen. The selection of strains was based on PHB content induced by nitrogen and phosphorus starvation. The fast-growing mutant MT_a24 exhibited more than 2.5-fold higher PHB productivity than that of the wild-type, attaining values of 37 ± 4 % dry cell weight PHB. The MT_a24 was characterized for phenotypes, CO_2 uptake rate and gene expression levels using quantitative PCR. Genome sequencing showed that UV mutagenesis treatment resulted in a point mutation in the ABC-transport complex, phosphate-specific transport system integral membrane protein A (PstA). The MT_a24 shows potential for industrial production of PHB and also for carbon capture from the atmosphere or point sources.

This manuscript is published in the Journal of Bioresource Technology.

Kamravamanesh Donya, Kovacs Tamas, Pflügl Stefan, Druzhinina Irina, Kroll Paul, Lackner Maximilian, Herwig Christoph. Increased poly-β -hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: Mutant generation and characterization. Bioresource Technology 266. 2018: 34-44.

Bioresource Technology 266 (2018) 34–44

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Increased poly-β-hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: Mutant generation and characterization

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ARTICLE INFO

Keywords:
Poly-β-hydroxybutyrate (PHB)
UV-mutagenesis
Synechocystis sp. PCC 6714
CO_2 uptake rate
Inorganic phosphate system transport (Pst)

ABSTRACT

Photosynthetic Poly-β-hydroxybutyrate (PHB) productivity in cyanobacteria needs to be increased to make cyanobacterial derived bioplastics economically feasible and competitive with petroleum-based plastics. In this study, high PHB yielding mutants of *Synechocystis* sp. PCC 6714 have been generated by random mutagenesis, using UV light as a mutagen. The selection of strains was based on PHB content induced by nitrogen and phosphorus starvation. The fast growing mutant MT_a24 exhibited more than 2.5-fold higher PHB productivity than that of the wild-type, attaining values of 37 ± 4% dry cell weight PHB. The MT_a24 was characterized for phenotypes, CO_2 uptake rate and gene expression levels using quantitative PCR. Genome sequencing showed that UV mutagenesis treatment resulted in a point mutation in the ABC-transport complex, phosphate-specific transport system integral membrane protein A (PstA). The MT_a24 shows potential for industrial production of PHB and also for carbon capture from the atmosphere or point sources.

1. Introduction

Cyanobacteria are promising platforms for the photosynthetic production of poly-β-hydroxybutyrate (PHB) (Oliver et al., 2016). PHB is a promising biotechnological product from the class of bacterial polyhydroxyalkanoates (PHA), which are naturally occurring thermoplastic polyesters and can replace fossil-derived plastics in most applications, such as disposable bulk materials in packaging films, containers, and paper coatings and in biomedical applications (Fabra et al., 2015; Madison and Huisman, 1999). Life cycle assessment studies have suggested that using PHB as a replacement of conventional petrochemical polymers lowers environmental impacts (Pietrini et al., 2007) and also can reduce marine littering and microplastics pollution (Lackner, 2015).

Other than their potential for reducing greenhouse gas emissions and reducing production costs, cyanobacteria have natural pathways for the production of carbohydrates and proteins as well as metabolites such as vitamins and carotenoids (Ruffing and Kallas, 2016; Wang et al., 2014). However, from an economic point of view, photosynthetic PHB production in cyanobacteria has a major drawback, as the native PHB content is normally very low (< 10% dry cell weight (DCW) (Drosg, 2015). Attempts to obtain wild- type high PHB producing cyanobacterial strains have rarely been successful. So far the thermophilic cyanobacterial strain *Synechococcus* sp. MA-19 with 55% (DCW) PHB (Nishioka et al., 2001) is the highest reported amount of PHB from CO_2. Cyanobacterial strain improvement to increase PHB productivity from CO_2 is an important step to enable a feasible production of this biodegradable polymer (Balaji et al., 2013).

Even though the mechanism by which PHB is produced in cyanobacteria is not completely understood, the main accepted hypothesis is that when cyanobacteria are exposed to unfavorable growth conditions, like nitrogen and phosphorus limitation, the excess energy or the disproportion in the ratio of carbon: nitrogen or NADPH: ATP is effectively directed towards carbon reserves such as PHB and glycogen synthesis as

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https://doi.org/10.1016/j.biortech.2018.06.057
Received 19 April 2018; Received in revised form 18 June 2018; Accepted 19 June 2018
Available online 20 June 2018

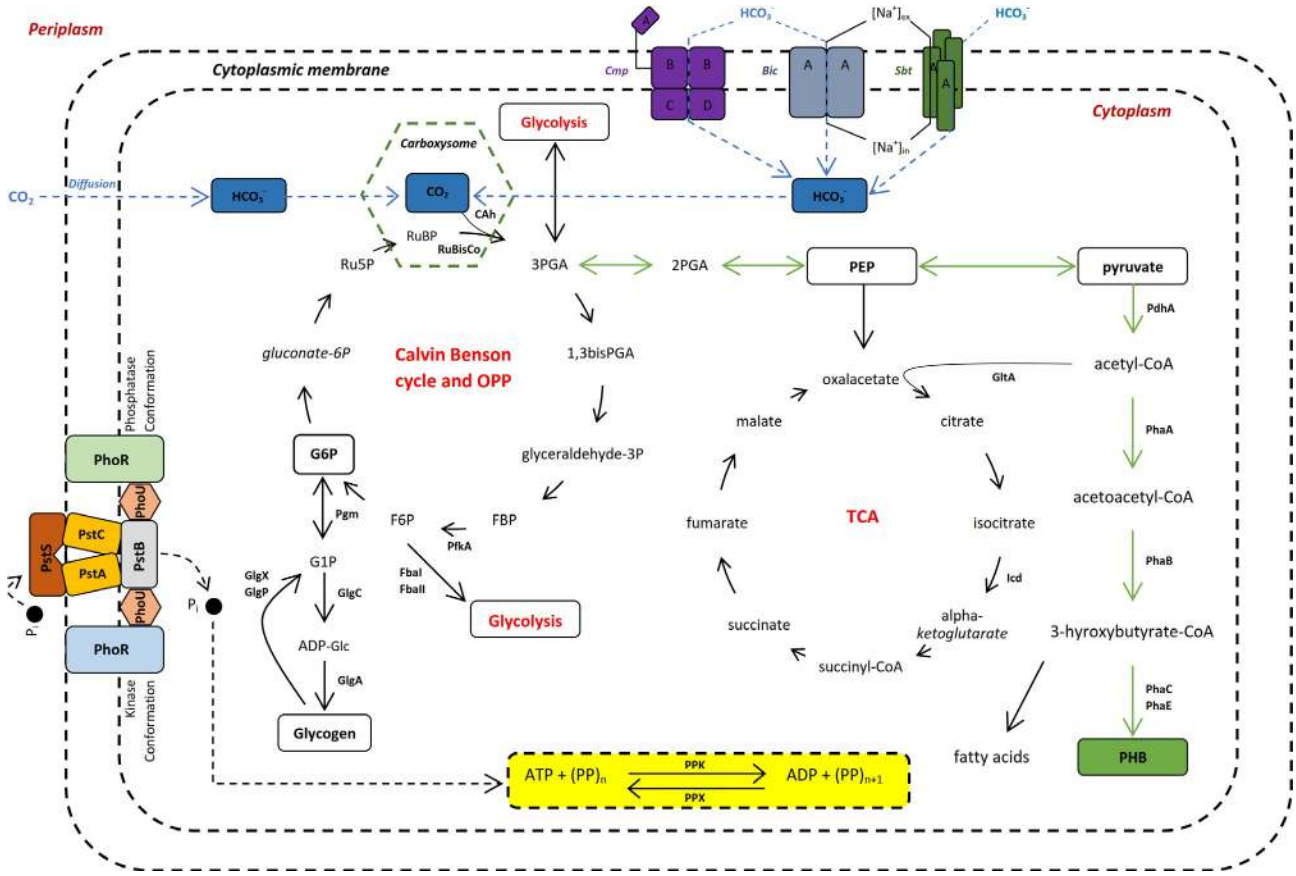

Fig. 1. Simplified biosynthetic pathways for polyhydroxybutyrate (PHB) and glycogen production in cyanobacteria. The pathways are reproduced from the previously shown data by (Burnap et al., 2015; Gao et al., 2012; Vuppada et al., 2018) with some modifications. Two possible routes for the PHB synthesis are shown i) direct carbon dioxide fixation via the Calvin-Benson cycle, producing sugar phosphates and conversion of pyruvate to acetyl-CoA (indicated by green arrows) and ii) via the glycogen oxidation by glycogen catabolic enzymes back to the Calvin-Benson cycle. Not all reactions are given, and the enzymes in Calvin-Benson cycle and tricarboxylic acid (TCA) cycle are not fully shown. **Abbreviations**: Ru5P, ribulose-5- phosphate; RuBP, Ribulose-1,5- bisphosphate; 3PGA, 3-phosphoglyceric acid; 1,3 bisPGA, 1,3-Bisphospho glyceric acid; FBP, Fructose-1,6- bisphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G1P, Glucose-1-phosphate; ADP-Glc, ADP-glucose pyrophosphorylase; OPP, Oxidative Pentose Phosphate pathway; PEP, Phosphoenolpyruvate; pp, polyphosphate; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; CAh, Carbonic anhydrase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reducing equivalents (De Philippis et al., 1992). Cyanobacteria as indicated in Fig. 1 fix atmospheric carbon dioxide via the Calvin-Benson cycle, producing carbon backbones; 3-phosphoglycerate for production of metabolites such as glycogen and PHB in some species (Wang et al., 2013). This metabolite is later utilized to provide the necessary carbon backbone for biosynthesis of proteins and other metabolites required for cell growth when essential nutrients are available (Hauf et al., 2015). PHB is synthesized in three biosynthetic steps where acetoacetyl-CoA is formed from two molecules of acetyl-CoA by the enzyme β-ketothiolase (encoded by *phaA*) (Hauf et al., 2015). In the second step, PhaB (encoded by *phaB*) reduces acetoacetyl-CoA to hydroxybutyryl-CoA, utilizing NADPH as the electron donor (Taroncher-Oldenburg et al., 2000). In the final step of biosynthesis, D-3-hydroxybutyryl-CoA is polymerized to PHB by a PHA synthase, comprising PhaC and PhaE (Hauf et al., 2015; Hein et al., 1998). Efforts to overcome bottlenecks in PHB synthesis pathways, including upregulation of native biosynthetic genes for PHB production namely phaA, B, C and E in *Synechocystis* sp. PCC 6803 in spite of a two-fold higher PHB content (26% DCW) (Khetkorn et al., 2016), could not yield higher productivity in terms of produced PHB per unit of time and reactor volume. Also the heterologous expression of *Cupriavidus necator* (Betaproteobacteria) PHB synthesis genes in *Synechocystis* sp. PCC 6803 (Sudesh et al., 2002) showed an increased activity of PHB synthase but was not associated with a significant increase in PHB levels. Conversely, lower expression of PHB synthesis genes has been reported in genetically engineered *Synechocystis* sp. PCC 6803 with high PHB accumulation (Lau et al., 2014). These observations suggest that PHB synthesis is not only regulated by the levels of enzymes of PHB synthesis, but by other factors such as intracellular metabolite concentrations and redox levels (Dutt and Srivastava, 2017). Since the directed approaches used so far could not lead to successful enhancement of PHB productivity in cyanobacteria, the application of an alternative technique seems crucial.

Mutagenesis, as a substitute approach to genetic engineering, has been used successfully to obtain improved bacterial strains as production hosts in biotechnology (Galanie et al., 2013). Several studies have been carried using microalgae and cyanobacteria to obtain desirable phenotypes using random mutagenesis. Cordero et al. (2011) showed enhancement of leutin production in *Chlorella sorokiniane* (Chlorophyta) by using N-methyl-Ń- nitro-nitrosoguanidine (MNNG) as a mutagen. Also, high triacylglycerol (TAG) yielding mutants of *Nannochloropsis* sp. (Ochrophyta) and *Scenedesmus obliquus* (Clorophyta) were obtained by random mutagenesis approach using ethyl methanesulfonate (EMS) and UV radiation respectively (de Jaeger et al., 2014; Doan and Obbard, 2012). Mutagenesis has also been used to promote phototolerance in the model cyanobacterial strain *Synechocystis* sp. PCC 6803 (Narusaka et al., 1999).

In this current study, the random mutagenesis was used as an effective tool to increase PHB production in the cyanobacterial strain

Synechocystis sp. PCC 6714. The strain can produce up to 16% (DCW) PHB under nitrogen and phosphorous limitation by controlling the process parameters and supply of CO_2 as the only carbon source (Kamravamanesh et al., 2017). The most promising mutants were compared to the wild-type strain in terms of biomass growth and PHB content under defined conditions of a multi-photobioreactor system. BIOLOG Phenotype Microarray (PM) assays were used as a method to check the fitness and to characterize the nitrogen and carbon metabolism in the wild-type strain *Synechocystis* sp. PCC 6714 and the best mutant MT_a24. Genome sequencing and quantitative PCR following mutagenesis were done to get more insight into the PHB accumulation mechanism of cyanobacteria and to define targets for future strain engineering.

2. Material and methods

2.1. Strain and mutant generation

An axenic culture of wild-type strain *Synechocystis* sp. PCC 6714 was purchased from Pasteur Culture Collection of Cyanobacteria (Pasteur Institute, Paris, France). Unless stated otherwise, *Synechocystis* sp. PCC 6714 was grown in BG-11 medium (Rippka et al., 1979) supplemented with 10 mM HEPES buffer pH 8.2 with the addition of 15 g L^{-1} of Kobe agar for plates and 5 mM $NaHCO_3$ as carbon source prior to inoculation. In order to induce nitrogen deficiency, cells were cultured in BG-11 media without nitrate and ammonia. Ferrous ammonium citrate and Co $(NO_3)_2{\cdot}6H_2O$ were substituted with equimolar concentrations of Ferric citrate and $CoCl_2{\cdot}6H_2O$ in terms of iron and copper content. For phosphorus limitation, KH_2PO_4 was replaced with an equimolar concentration of KCl in terms of potassium content.

In total, approximately 10^4 cells from late log phase were plated on agar plates and were incubated at 28± 2 °C under continuous illumination with 50± 5 µmol photon m^{-2} s^{-1} in photosynthetically active radiation (PAR) in an incubator from Infors (Switzerland). Once colonies were visible, the plates were exposed to monochromatic UV light with a wavelength of 254 nm at room temperature. After irradiation, the plates were stored in dark conditions for at least 72 h to reduce light-induced repair mechanisms. Subsequently, the plates were incubated under continuous illumination for 120 h. The mutated colonies were removed from plates and cultivated in liquid media on 6-well plates from Greiner CELLSTAR® (Merk, Austria). After 96 h of incubation, the colonies which showed superior growth when compared to the wild type were screened by measuring OD_{750} using an infinite 200 microplate reader (TECAN, Switzerland). The cells were harvested by centrifugation at 5000*g* at room temperature and were re-suspended in media without nitrogen and phosphorus. The PHB content was determined after 4 and 7 days of limitation. The colonies which showed maximum PHB content after 7 days of limitation were selected for further studies.

2.2. Growth determination and estimation of dry weight

Biomass growth was determined spectrophotometrically at 750 nm using a UV–Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 24-hour intervals. Dry cell weight (DCW) was determined in triplicates by transferring 10 mL of fermentation broth or shake flask cultures to a reusable pressure filter holder (Sartorius, Göttingen, Germany) and by filtering on a pre-weighed 0.45 µm Cellulose acetate filter paper (Sartorius, Göttingen, Germany) at a pressure of 6 bar for 1 min. Filters were dried overnight at 70 °C and dry weight was determined gravimetrically. A correlation between optical density and DCW could be established, taking into account that this correlation changes over cultivation time and is described in Eq. (1). Cx represents the biomass concentration in g L^{-1}.

$$C_x = 0.37 \times OD_{750} \cdot [g\ L^{-1}] \quad (1)$$

2.3. Bioreactor cultivations

Bioreactor experiments were carried out under sterile conditions in a DASbox Mini Bioreactor System (Eppendorf AG, Hamburg, Germany) with a maximum working volume of 250 mL. The two step cultivations were performed first by allowing the cells to grow on complete BG-11 media for 135 h. The limitation phase started by harvesting the cells using centrifugation at 3000 rpm at room temperature and transferring them back into the reactors containing 250 mL of BG-11 media without nitrogen and phosphorous source. The pH was maintained at 8.5 by addition of 0.5 M HCl or NaOH added to the reactors with a DASGIP MP, multi-pump module (Eppendorf AG, Germany). The agitation was set at 300 rpm and the reactors were bubbled with a mixture of sterile filtered air and 2% CO_2 at a flow rate of 0.4 vvm (6 L h^{-1}). The illumination was done using LED strips wrapped around the reactor vessels providing a light intensity of 60 µmol m^{-2} s^{-1} photons in PAR. The five M18 ports were occupied by pH, DO120 sensor (Hamilton, Reno, NV, USA), OD sensor (Eppendorf DASGIP OD4 Module, 880 nm), and gas inlet and a gas outlet. The exhausted gas was analyzed by a DASGIP GA4 gas sensor module (Eppendorf AG, Hamburg, Germany) with a ZrO_2 sensor for O_2 and infrared CO_2 sensor technology.

All fermentation parameters and variable pump set-points were controlled using the DASware control system (Eppendorf AG, Hamburg, Germany).

Photobioreactor experiments were performed in biological duplicates. Samples were taken in triplicates at 24-hour intervals and were analyzed for dry cell weight, glycogen, and PHB content.

2.4. Determination of glycogen content

Glycogen quantification was done using a protocol from Forchhammer and Tandeau de Marsac (1995) with some modifications. Pre-weighed dried cells from 2 mL culture were heated with 1 mL of 7.5% v/v of the sulfuric acid solution at 95 °C on a heating block (Accublock™, Labnet, USA) for 120 min. Glucose was produced from glycogen by acid hydrolysis, the hydrolysate was then quantified by ion chromatography using the method explained by Hofer et al. (2018). For calibration, pure glycogen (Sigma- Aldrich, USA) was treated accordingly and analyzed in parallel with samples. The method was run on an Ion chromatography ICS-5000 (ThermoFisher Scientific, USA), equipped with a pump (LPG), an autosampler (AS-AP) with a 25 µL sample loop, a detector compartment (DC) and an electrochemical detector. Chromeleon 7.2 was used for the control of the devices as well as for the quantification of the peak areas. The glycogen content per (DCW) was calculated as explained in equation (2).

$$\%\ \text{DCW Gly} = \frac{\text{mg(Gly)}}{\text{mg(DCW)}} * 100. \quad (2)$$

2.5. Determination of the PHB content

PHB quantification was done using the procedure described by Schlebusch and Forchhammer (2010). Pre-weighed dried cells (2–5 mg) were boiled with 1 mL conc. H_2SO_4 at 100 °C on a heating block (Accublock™, Labnet, USA) for one hour to convert PHB to crotonic acid. Samples were allowed to cool down and subsequently diluted 20 times using 0.014 M H_2SO_4. Crotonic acid was determined using a high-performance liquid chromatography system (Thermo-Fischer Scientific, USA) with a Nucleosil C8 column (Macherey-Nagel, Germany) using an isocratic method. The mobile phase used was 20 mM NaH_2PO_4 buffer; pH 2.5 and acetonitrile (70:30 v/v) with a flow rate of 0.85 mL min^{-1} and a column temperature of 30 °C. Detection of crotonic acid was done using a diode array detector (DAD) detector (Thermo-Fischer Scientific, USA) at 210 nm. For calibration, pure PHB (Sigma- Aldrich, USA) was treated accordingly and analyzed in parallel with samples. Instrument

control and peak evaluation were done with Chromeleon 7.2 (ThermoFischer Scientific, USA). The percentage PHB per (DCW) was determined by the amount of PHB obtained from HPLC analysis and the cell dry weight of biomass used for the analysis using Eq. (3):

$$\%\text{DCW PHB} = \frac{mg(\text{PHB})}{mg(DCW)} * 100. \quad (3)$$

2.6. Phenotyping assay

The phenotyping assay was performed using the Biolog microplates PM1 and PM3B (Biolog, California, USA) for carbon and nitrogen metabolism, respectively. For the carbon assays the cells were suspended into 1:10 diluted BG-11 media with biomass concentration of $OD_{750} = 0.1$. For the nitrogen assays the cells were suspended in BG-11 media with no nitrogen source and 5 mM $NaHCO_3$ as the carbon source. The 90 µL of cell suspension were inoculated into each well and the plates were incubated at 28 °C under 150 rpm agitation and continuous illumination with 30 µmol m^{-2} s^{-1} in PAR in the shaking incubator (Infors, Switzerland). Growth was monitored daily using OD measurements at 750 nm for 168 h, results were analyzed in Statistica 6.0 (StatSoft, USA).

2.7. DNA extraction and genome sequencing

DNA extraction was done for the wild-type strain *Synechocystis* sp. PCC 6714 and the mutant MT_a24 using the DNeasy UltraClean Microbial Kit (QIAGEN, CA, USA) according to the manufacturer's recommendation. The purified DNA pellet was re-suspended in DNase free MQ water, quantified using a Nanodrop 1000 (ThermoFisher Scientific, USA) and stored at −80 °C.

The genome sequencing was done using Illumina Nextera XT libraries on Illumina NextSeq by Microsynth Austria GmbH (Vienna, Austria). The genome was assembled, annotated and the detection of large modifications in the genome was determined using the CLC Genomics Workbench (Qiagen Bioinformatics, CA, USA).

2.8. RNA extraction and quantitative PCR

The cells (25–30 mL) from late exponential phase were harvested by centrifugation at 14000 rpm for 5 min at 0 °C and were stored in liquid nitrogen immediately after sampling. Frozen cells were homogenized in 3 mL lysis buffer, and RNA was isolated using the PureLink RNA Mini Kit (Ambion by life technologies, ThermoFisher Scientific, USA) according to the manufacturer's recommendation. DNA digestion was performed on a column containing RNase-free DNase (On-column DNAase I Digestion Set, Sigma, Austria). The purified RNA pellet was re-suspended in RNase free MQ water and was quantified using a Nanodrop 1000 (ThermoFisher Scientific, USA).

The RNA was reverse transcribed using the RevertAid™ Reverse Transcriptase kit (ThermoFisher Scientific, USA) using 200 ng of total RNA according to manufacturer's protocol with a combination of the provided oligo (dT) and random hexamer primers (20 µL). The quality of the cDNA fragments was analyzed using the Fragment analyzer system (Advanced Analytical Technologies, USA).

Gene expression levels were determined by gene-specific quantitative real-time PCR using Luna Universal qPCR Master Mix (New England Biolabs, USA) and 100 nM primers in the system. The sequences of the forward and reverse primers are provided in (supplementary data). The Cytochrome b6-f complex subunit gene, RNA subunit of ribonuclease P, and a small subunit of ribosomal RNA which maintained constant overall expression (± 20 relative %) both under normal growth conditions and under nitrogen and phosphorus limitation was used as the normalizer. The specification of the PCR was determined using a dissociation stage on a qTower 2.2 (Analytik Jena AG, Germany) system. Determination of primer efficiency was performed using triplicate reactions from a dilution series of cDNA (1, 0.1 and 10^{-2}) for the housekeeping genes mentioned above. For the results, the mean Ct values were determined using the method from (Bustin, 2004) by calculating the average of the triplicate measurements for each species and gene. The ΔCt was calculated by subtracting the mean Ct value of the housekeeping gene from the mean Ct value of the gene of interest. ΔΔCt is constituted by the difference between the mutant sample Ct and the wild-type sample as control Ct values. Finally, the relative quantity, which has been shown in this manuscript was calculated by applying the following equation:

$$\text{Relative quantity} = 2^{-\Delta\Delta Ct} \quad (4)$$

2.9. Statistical analysis

Unless otherwise stated experiments were performed in biological triplicates. Error bars are represented as mean ± standard deviation (SD). Statistical analysis was performed with two tailed Student T-test whereas P values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Mutant development and screening

Considering strain improvement as a function of growth and PHB production, an accelerated natural evolution approach was used employing UV mutagenesis. At first, the length and distance from the UV light were optimized in terms of viability. Over 2000 mutants were generated after UV irradiation in micro-well plate experiments. All mutants were screened for growth using the wild-type strain *Synechocystis* sp. PCC 6714 as the reference. 850 mutants with superior biomass concentration were further investigated for PHB accumulation. The % DCW PHB content was determined 4 and 7 days after the start of nitrogen and phosphorus limitation using the wild-type strain as a control. In total, 14 mutants showed significantly higher PHB content than the wild-type strain and therefore, were selected for further studies. As shown in Fig. 2 the mutants MT_a16 (31% DCW) and MT_a37 (30% DCW) showed up to 5-fold increase in PHB content compared to

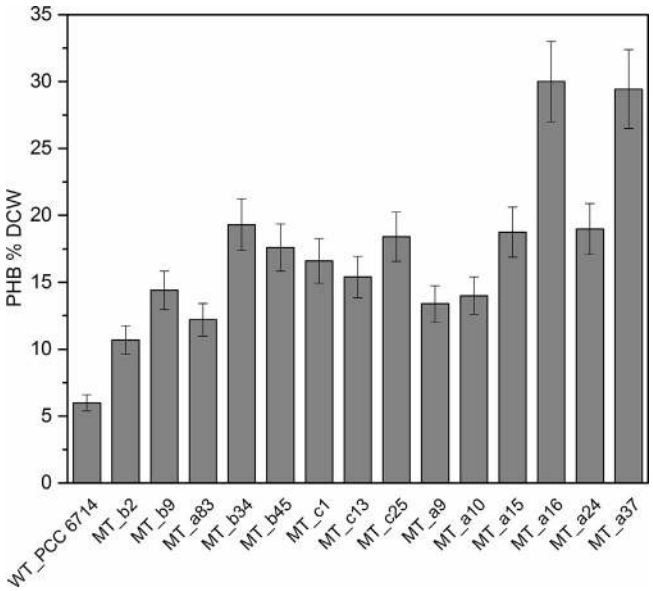

Fig. 2. PHB content of wild-type *Synechocystis* sp. PCC 6714 (WT_PCC 6714) and 14 selected mutants after 7 days of nitrogen and phosphorus limitation. Each data point is obtained from duplicate cultivations and error bars represent mean ± SD.

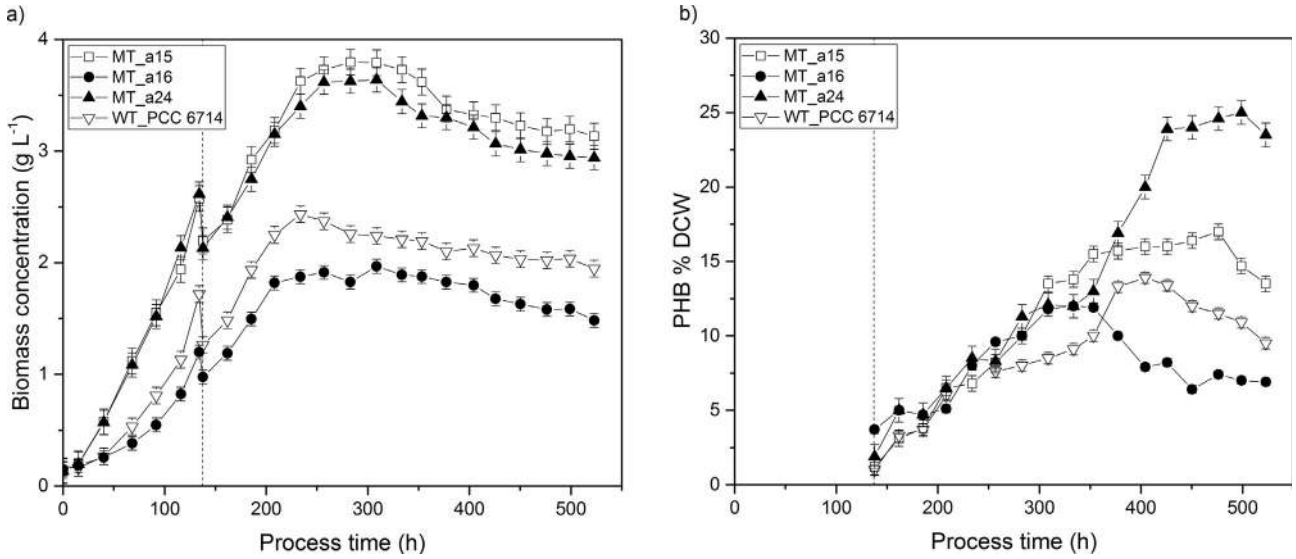

Fig. 3. Two-step cultivation of selected mutants and the wild-type strain *Synechocystis* sp. PCC 6714 in a multi-photobioreactor system a) growth curve for three mutants namely MT-a15, MT_a16, MT_a24 and the wild-type strain *Synechocystis* sp. PCC 6714. b) PHB content in % DCW for the three selected mutants namely MT-a15, MT_a16, MT_a24 and the wild-type PCC6714 strain under nitrogen and phosphorus limitation. The vertical line represents the start of the limitation phase. The values are derived from biological duplicate cultures. Deviation from the duplicate average is indicated by the error bars.

the wild-type *Synechocystis* sp. PCC 6714 with 6% (DCW) PHB after 7 days of nitrogen and phosphorus limitation. Mutants MT_b34, MT_c25, MT_a15 and MT_a24 with 20% (DCW) PHB showed 3.5 folds higher product content when compared to the wild-type. Moreover, as the selection experiments were performed in micro-well plates later during the scale up to shake flask cultivations only three mutants namely, MT_a24, MT_a15 and MT_a16 showed promising potential in terms of biomass growth and PHB production. For instance, the strain MT_a37 showed major defects after multiple cultivations resulting in inferior growth and PHB yield and subsequently was not used for further experiments. To this end, the three mutants MT_a24, MT_a15 and MT_a16 were selected for bioreactor cultivations.

3.2. Mutant characterization

3.2.1. Growth and PHB content

With the view to study growth kinetics and PHB productivity of three selected mutants and the wild-type strain *Synechocystis* sp. PCC 6714, a two-step cultivation was done under controlled defined conditions in a multi-photobioreactor system. The results which are shown in Fig. 3a indicate superior biomass growth for two mutants, namely MT_a24 and MT_a15 both under normal as well as limiting growth conditions when compared to the wild-type. The maximum biomass concentration for mutant MT_a24 was 3.6 ± 0.4 g L^{-1} after 283 h of the process and 3.8 ± 0.4 g L^{-1} for mutant strain MT_a15, which were respectively 1.5 and 1.6 times higher than the wild-type with a maximum biomass concentration of 2.4 ± 0.2 g L^{-1}. The mutant MT_a16 with 2 ± 0.2 g L^{-1} achieved the lowest biomass concentration. The doubling time determined for the mutants MT_a24 and MT_a15 were respectively 16.2 and 16.4 h which are much lower than the doubling times obtained for the mutant MT_a16 and wild-type strain with 40 and 27.9 h. Analysis of the PHB content of the studied strains showed that the highest PHB content of 25 ± 0.7% (DCW) was obtained after 500 h for MT_a24 and 17 ± 0.8% (DCW) for MT_a15 while the wild-type strain reached at 14 ± 0.5% (DCW) PHB after 405 h of nitrogen and phosphorus limitation (Fig. 3b). The maximum polymer concentration was obtained for the mutant MT_a24 with highest PHB content of 735 ± 28 mg L^{-1} after 426 h followed by mutant MT_a15 with 530 ± 20 mg L^{-1} after 476 h. The PHB content obtained for the MT-a24 and MT_a15 were 2.5 and 1.8 times higher, respectively, when compared to that of the wild-type strain. The maximum PHB content for the wild-type strain *Synechocystis* sp. PCC 6714 was 297 ± 15 mg L^{-1} which in turn was higher than that of the mutant MT_a16 with 233 ± 12 mg L^{-1}.

The specific growth rates, average, and maximal volumetric biomass productivity, as well as the volumetric PHB productivity, are shown in Table 1. Under nitrogen and phosphorus replete growth, MT_a24 had the highest average biomass productivity compared to the wild-type and the other two mutants, MT_a15 and MT_a16, with the value of 413 ± 40 mg L^{-1} d^{-1}. However, under nitrogen and phosphorus limiting conditions, MT_a15 showed the highest average biomass productivity of 66 ± 7 mg L^{-1} d^{-1} when compared to the wild-type followed by the MT_a24 with 54 ± 5 mg L^{-1} d^{-1}. The highest maximum volumetric biomass productivity both for growth and limitation phase was obtained for the MT_a15 with 636 ± 64 mg L^{-1} d^{-1} and 537 ± 54 mg L^{-1} d^{-1} respectively. Also, the MT_a24 and MT_a15 obtained the highest average specific growth rate of 0.5 d^{-1}. However, the maximum specific growth rate was slightly higher for mutant MT_a24 than MT_a15 with values of 1.07 and 1.05 d^{-1} which are one and half-fold higher than for the wild-type strain with a maximum specific growth rate of 0.7 d^{-1}. The highest average volumetric PHB productivity was obtained for mutant MT_a24 with the value of 36.9 ± 4 mg L^{-1} d^{-1} which was at least three-fold higher than that of the wild-type strain with 11 ± 1 mg L^{-1} d^{-1}. The highest maximum volumetric PHB productivity was obtained respectively for MT_a15 with 173.7 ± 17 mg L^{-1} d^{-1} and MT_a24 with 134.2 ± 13 mg L^{-1} d^{-1}.

3.2.2. Carbon uptake rate and the link to PHB productivity

The earlier results showed superior volumetric biomass and PHB production for two mutant strains of *Synechocystis* sp. PCC 6714, MT_a15 and MT_a24. These observations encouraged the determination of CO_2 uptake rate and the possible correlation to PHB production. As it could be expected the higher net CO_2 consumption was obtained for mutants MT_a24 followed by the MT_a15 with values of 1140 and 900 mmol, respectively (Fig. 4a). These values are 4 and 3 fold higher, respectively than the values obtained for the wild-type (277 mmol) and the mutant MT_a16 (238 mmol). In order to evaluate whether the higher CO_2 consumption in MT_a24 and MT_a15 was due to higher biomass concentration or other unknown genetic or metabolic

Table 1
Volumetric productivities and the specific growth rates of *Synechocystis* sp. PCC 6714 and three selected mutants. The maximum specific growth rate is given as the highest value observed between two daily sampling points. The average and the maximum specific growth rates shown were only determined for the growth phase (0 – 135 h). The average productivities are calculated as the amount of biomass and PHB formed over the course of time per liter of culture media. The maximal productivities represent the highest production values between two consecutive time points. Data represent mean ± SD of biological duplicate cultures. Bold numbers represent the highest number for each parameter.

Strain	Media	Volumetric biomass productivity mg L^{-1} d^{-1}		Specific growth rate d^{-1}		Volumetric PHB productivity mg L^{-1} d^{-1}	
		Average	Maximum	Average	Maximum	Average	Maximum
WT_PCC 6714	N^+P^+	262 ± 30	588 ± 60	0.41	0.70		
	N^-P^-	15 ± 1	451 ± 45			11.0 ± 1	101.6 ± 10
MT_a24	N^+P^+	**413 ± 40**	618 ± 60	**0.50**	**1.07**		
	N^-P^-	54 ± 5	401 ± 40			**36.9 ± 4**	134.2 ± 13
MT_a15	N^+P^+	406 ± 40	**636 ± 64**	**0.50**	1.05		
	N^-P^-	**66 ± 7**	**537 ± 54**			26.4 ± 3	**173.7 ± 17**
MT_a16	N^+P^+	170 ± 17	377 ± 38	0.35	0.41		
	N^-P^-	40 ± 5	323 ± 32			7.14 ± 0.7	84.6 ± 8

The N^+P^+ represents growth when sufficient media components were available (growth phase, first 135 h of the cultivation)
The N^-P^- represents growth under nitrogen and phosphorous limiting conditions (from 135 h until the end of the cultivation).

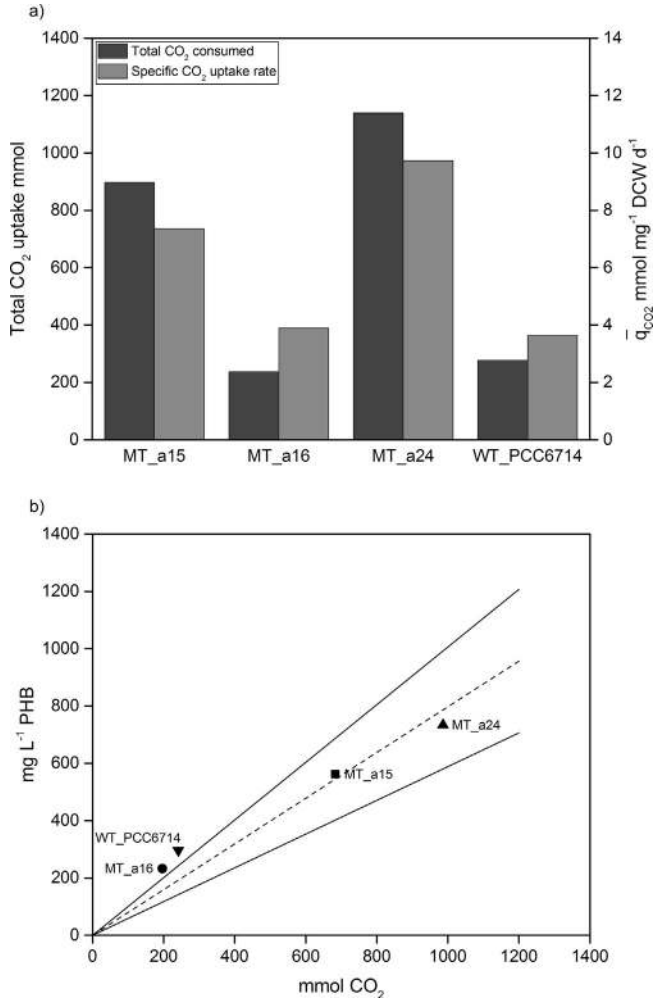

Fig. 4. The net consumed CO_2 (in mmol), the average specific CO_2 uptake rate and correlation to PHB formation are shown. (a) The total net CO_2 consumed and the average specific CO_2 uptake rate in mmol CO_2 mg^{-1} DCW d^{-1} are shown for three mutants MT_a15, MT_a16, MT_a24, and the wild-type strain *Synechocystis* sp. PCC 6714. (b) The results of the linear regression analysis of net CO_2 consumption and maximum PHB produced are shown for a two-step cultivation. The model obtained shows statistically significant parameters with the coefficient of determination, $R^2 = 0.98$ and p value of 0.001 with an upper and lower 95% limit of 1.0 and 0.59, respectively. The experiments were done in biological duplicate cultures and one cultivation is shown as an example.

alterations, the specific CO_2 uptake rate was determined in mmol CO_2 consumed per mg biomass generated per day. Besides net CO_2 consumption, the average specific uptake rates of CO_2 are higher for the mutants with higher PHB productivity namely MT_a24 (9.7 mmol mg^{-1} d^{-1}) and MT_a15 (7.4 mmol mg^{-1} d^{-1}) compared to the WT_PCC 6714 (3.7 mmol mg^{-1} d^{-1}) and MT_a16 (3.8 mmol mg^{-1} d^{-1}) (Fig. 4a). In order to correlate CO_2 uptake of the respective strain with PHB production, linear regression analysis was performed using the highest PHB content obtained for each individual strain during limitation phase and the CO_2 consumed up to that time point. As shown in Fig. 4b the CO_2 uptake directly correlates with PHB production for the two strains MT_a24 and MT_a15. Strains MT_a16 and PCC 6714 are out of 95% interval showing slightly lower CO_2 consumption than the amount of PHB which was produced which could be due to differences in PHB yield or simply other unknown mechanisms. The slope of the linear regression line in Fig. 4b represents the product yield $Y_{PHB/CO2}$, which is 0.80 ± 0.21. The data obtained here are consistent with our previous observations. The mutant MT_a24 shows superior volumetric biomass and PHB productivity, as well as higher specific CO_2 uptake rate compared to the other mutants and the wild-type strain. Hence, mutant MT_a24 was used hereafter for detailed examination and further experiments.

3.2.3. Phenotyping Microarray

The ultimate goal in biotechnological strain improvement is to obtain desirable phenotypes such as high productivity. As a result of random mutagenesis in MT_a24, the volumetric productivities and CO_2 uptake rate were increased. Hence, evaluation of the effect of the mutation on carbon and nitrogen assimilation behavior was performed. Therefore, the mutant MT_a24 was compared to its parent, wild-type strain *Synechocystis* sp. PCC 6714 using carbon and nitrogen Biolog PM assays. The Biolog PM assay has been used for metabolic phenotyping of various microbial species including bacteria, fungi and also microalgae (Pinzari et al., 2016). However, it has not been reported for the phenotyping of cyanobacteria. The complete growth curve was generated for each nutrient source. Table 2 shows the maximum specific growth rate and the average biomass concentration for statistically significant nitrogen and carbon sources after 144 h of cultivation where the difference in OD_{750nm} of the wild-type and mutant MT_a24 was higher than 0.15 and the p values < 0.05. In general, under normal growth conditions when nitrate was used as nitrogen source the MT_a24 grew similar or superior to the wild-type on most carbon sources. The best carbon sources for the growth of MT_a24 were D-Pscicose and glycyl-L-glutamic acid with an OD_{750nm} of 0.53 and 0.54, respectively. The best nitrogen sources for the MT_a24 were identified to be nitrate

Table 2
Average biomass growth on statistically significant carbon and nitrogen sources from Biolog PM Assays. The average biomass concentration (OD_{750}) and maximum specific growth rate (μ_{max}) are given for the statistically significant carbon and nitrogen sources for wild-type strain *Synechocystis* sp. PCC 6714 (1) and the best mutant MT_a24 (2). The standard deviation and P values are given. The carbon and nitrogen sources which have significantly higher growth in mutant MT_a24 are given in bold.

WT_ PCC 6714 = 1 MT_a24 = 2	1 $\mu_{max}(d^{-1})$	1 Average biomass conc. OD_{750}	1 SD	2 $\mu_{max}(d^{-1})$	2 Average biomass conc. OD_{750}	2 SD	P value
Carbon sources							
Glycyl-L-glutamic acid	0.048	**0.34**	0.03	**0.240**	**0.54**	0.18	0.002
Glycin-L-proline	0.024	0.25	0.01	0.048	0.34	0.03	0.001
Phenylethylamine	0.024	0.17	0.02	0.072	0.26	0.05	0.007
Inosine	0.024	0.24	0.01	0.024	0.34	0.02	< 0.001
D-glucose-1-phosphate	0.024	0.26	0.02	0.072	0.41	0.10	0.011
D-glucose-6- phosphate	0.024	0.20	0.02	0.168	0.29	0.12	< 0.001
N-acetyl-D-glucosamine	0.004	0.15	0.01	0.048	0.23	0.05	0.011
L-arabinose	0.024	0.12	0.04	0.048	0.18	0.02	0.016
D-Pscicose	**0.072**	**0.35**	0.10	**0.192**	**0.53**	0.11	0.028
Bromo succinic acid	0.024	0.26	0.01	0.120	0.46	0.10	0.008
Propionic acid	0.024	0.23	0.01	0.024	0.25	0.03	< 0.001
D-gluconic acid	0.005	0.15	0.01	0.072	0.23	0.06	< 0.001
m- tartaric acid	**0.003**	0.20	0.07	0.192	0.30	0.05	0.025
m- hydroxy phenyl acetic acid	0.024	0.29	0.04	0.096	0.37	0.08	0.044
D- galacturonic acid	0.024	0.15	0.02	0.096	0.27	0.07	0.004
Control (No carbon source)	< 0.001	0.12	0.01	0.001	0.16	0.01	0.001
Nitrogen sources							
Nitrite	0.048	**0.18**	0.07	**0.216**	**0.40**	0.18	0.036
Nitrate	**0.072**	0.18	0.07	0.096	0.32	0.11	0.039
Control (No nitrogen source)	< 0.001	0.08	0.01	0.001	0.11	0.01	0.004

Maximum specific growth rate and the average biomass concentration were determined for statistically significant nitrogen and carbon sources after 144 h of cultivation where the difference in OD_{750nm} between the wild-type and mutant MT_a24 was higher than 0.15, corresponding to a p value of < 0.05.

and nitrite with maximum OD_{750nm} of 0.40 and 0.32, respectively. For the mutant MT_a24, rapid growth, as well as the ability to assimilate diverse substrates as carbon and nitrogen source, is a great advantage as it suggests a high degree of fitness of the strain.

3.2.4. Stability of the mutation

The mutant MT_a24 generated in this study was regularly screened for the stability of the mutation over multiple generations, as the mutant does not contain a selection marker or any other trait to apply an external selection pressure to maintain the mutation. The results obtained from four individual two-step cultivations of MT_a24 over the course of 500 h is presented in Fig. 5. The biomass growth curves displayed in Fig. 5a shows that the maximum volumetric biomass concentration obtained for all cultivations was 4.6 ± 0.2 g L^{-1}. A similar pattern for growth has been observed both during growth and limitation of nitrogen and phosphorus. For all the cultivations of MT_a24, PHB and glycogen were accumulated simultaneously during the beginning of the limitation phase. The glycogen content reached a maximum of 50 ± 2% (DCW) after 73 h of nitrogen and phosphorous limitation (200 h from the start of the process) (Fig. 5b and c). Glycogen levels started to decline once the maximum level was reached while PHB content increased further to a maximum of 37 ± 4% (DCW) after approximately 450 h of cultivation (14 days of nitrogen and phosphorus limitation). The data obtained show the stability of the mutant MT_a24 over the course of 10 months. The strain MT_a24 was therefore deposited at National Collection of Microorganisms Cultures (CNCM)-Pasteur Institute (Paris, France) with the registration number (I-5255).

3.3. Genome sequencing and quantitative real-time PCR

3.3.1. Genome sequencing

The mutant strains were selected after physical mutagenesis solely based on improved product formation, while the biochemical or genetic nature of the mutation remained unknown. In order to elucidate the genetic basis causing the superior phenotype of strain MT_a24, whole genome sequencing of both mutant MT_a24 as well as wild-type *Synechocystis* sp. PCC 6714 used for the mutation experiments was performed. The sequence of the reads was mapped to the published genome of *Synechocystis* sp. PCC 6714 (Kopf et al., 2014). The sequenced genome of the mutant MT_a24 was deposited at National Center for Biotechnology Information with the accession number (SRP149881). A comparison of the two sequenced strains *Synechocystis* sp. PCC 6714 wild-type strain and mutant MT_a24 showed that surprisingly, only one mutation causing an amino acid substitution was introduced into the genome of MT_a24. The single mutation was found in an ABC transporter system, exhibited in (Supplementary data). The missense mutation which has resulted in the substitution of a single amino acid alanine (A) to proline (P) is located in the *PstA* gene from the inorganic phosphate-specific transport system (Pst). The Pst system constitutes of a periplasmic inorganic phosphate (P_i)- binding protein (PstS), two integral inner membrane proteins (PstA and PstC) that form a transmembrane channel which transport Pi through the cytoplasmic membrane, and an ATP-binding protein (PstB) (Surin et al., 1985). Besides transporting phosphate, the Pst system plays an important role in the regulation of a number of coordinately regulated genes that are phosphate repressible, of which the best known is *phoA*, the structural gene for alkaline phosphatase (Bachmann, 1983). The *pstA* gene encodes an integral membrane protein with six transmembrane helices (Surin et al., 1985), and its interruption reduces Pi transport, most likely by reducing the affinity of the protein and, hence, the Pst system, for insertion of P_i into the membrane (Rao and Torriani, 1990). Two copies of Pst systems, known as Pst1 and Pst2, have been identified in the genome of the cyanobacterial strain *Synechocystis* sp. PCC 6803 (Burut-Archanai et al., 2011). Both Pst systems encode ABC transporters and are upregulated during phosphate limiting conditions (Burut-Archanai et al., 2011). A mutation in Pst genes blocks Pi uptake under Pi-limited conditions (Cox et al., 1981). This may suggest a different level of phosphate transportation for the mutated strain MT_a24. To that end, the intracellular polyphosphate (PolyP) concentration of the wild-type strain *Synechocystis* sp. PCC 6714 and MT_a24 was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES). It has been shown that microorganisms store phosphorus in the form of PolyP when it is provided in excess (Rao et al., 2009). The PolyP storage form provides supplementary source of ATP when not enough

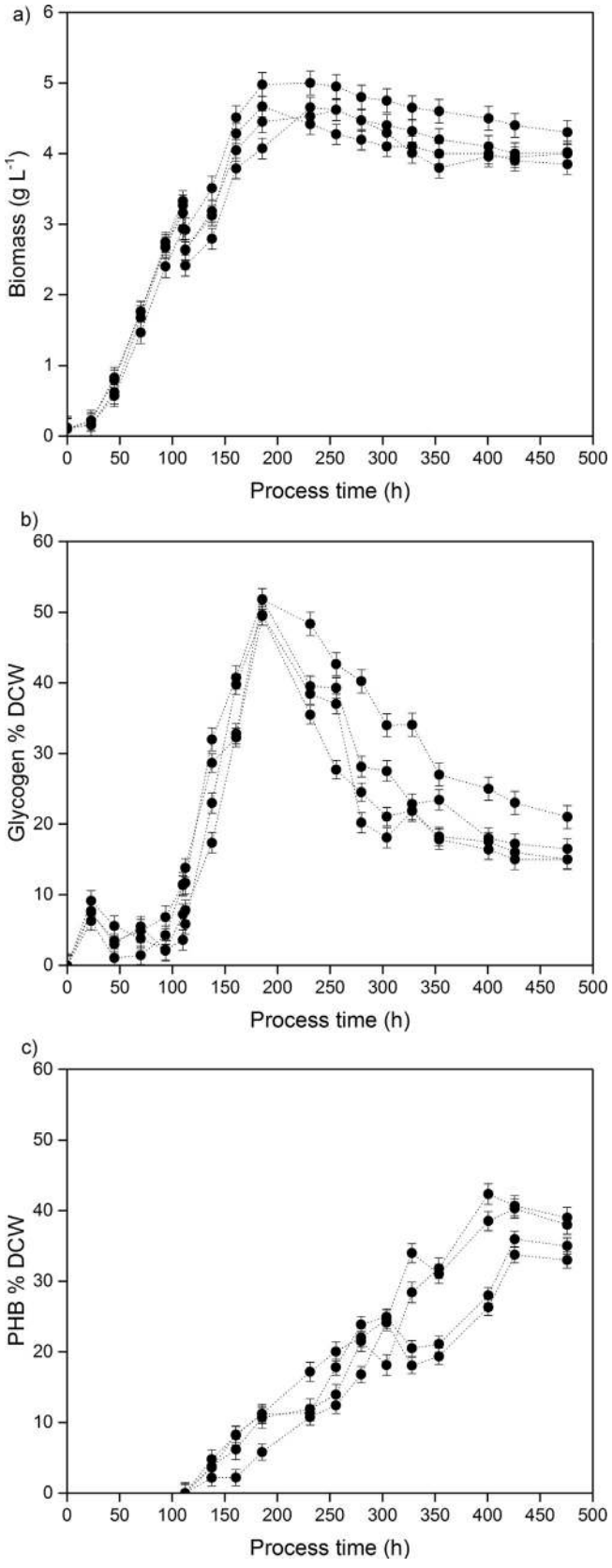

Fig.5. The mutant stability analysis using quadruplicate cultivations. (a) Biomass growth curves (b) percentage DCW glycogen content and (c) percentage DCW PHB content is shown for the quadruplicate cultivation of MT_a24 using a two-step process. The limitation of nitrogen and phosphorus was started at 115 h of the process. Data represent mean ± SD from three independent measurements.

ATP is produced by photosynthesis (Gomez-Garcia et al., 2013).

Surprisingly, the intracellular phosphorus concentrations showed similar levels for the mutant MT_a24 and the wild-type strain. For the sake of gaining insight into the gene expression levels of MT_a24 in comparison to the wild-type strain quantitative real-time PCR was performed for growth and nitrogen and phosphorus limiting conditions.

3.3.2. Quantitative real-time PCR

To study the effect of the mutation on gene regulation, quantitative real-time PCR analysis was performed checking the gene expression levels for the mutant MT_a24 and the parent strain *Synechocystis* sp. PCC 6714 during nitrogen and phosphorus limiting conditions. For the qPCR analysis of the wild-type strain *Synechocystis* sp. PCC 6714 and MT_a24 the genes involved in PHA biosynthesis, glycogen metabolism, bicarbonate, and phosphate transport system, as well as polyphosphate metabolism, were investigated. The analysis was performed on samples derived from 4 and 9 days of limitation. The relative expression levels of the studied genes after 9 days of nitrogen and phosphorus limitation are presented in Fig. 6. As a result of the mutation the expression level of eight genes, namely glycogen phosphorylase (*glgP*) (*p value = 0.045*), Bicarbonate transport system permease protein (*cmpB*) (*p value = 0.035*), bicarbonate binding protein (*cmpA*) (*p value = 0.040*), bicarbonate transport ATP-binding protein (*cmpC*) (*p value = 0.035*), large subunit of RuBisCO (*rbcL*) (*p value = 0.029*), exopolyphosphatase (*ppx*) (*p value = 0.050*), phosphoglucomutase (*pgm*) (*p value = 0.032*) and phosphate transporter protein C (*pstC*) (*p value = 0.043*) was increased in MT_a24 under nitrogen and phosphorus limiting conditions.

Upregulation of genes involved in bicarbonate transportation (*cmpA*, *cmpB*, *cmpC* and *sbtA*) was detected. This observation along with higher expression levels for *rbcL* supports our earlier results which have shown the superiority of mutant MT_a24 in terms of CO_2 uptake. The expression levels for PHA biosynthetic genes (*phaA*, *phaB*, *phaC,* and *phaE*) remain largely unchanged for the MT_a24 when compared to the wild-type strain. However, for the glycogen metabolic genes, namely glycogen phosphorylase (*glgP)* and phosphoglucomutase (*pgm*) expression levels exceeded those of the parental wild-type (WT- PCC 6714) under nitrogen and phosphorus limiting conditions.

In cyanobacteria the glycogen biosynthesis occurs via the phosphoglucomutase (Pgm) which catalyzes the first step of the interconversion of glucose-6P into glucose-1P, followed by the action of ADP-glucose pyrophosphorylase (GlgC) that synthesizes ADP-glucose, using glucose-1P and ATP (Diaz-Troya et al., 2014; Xu et al., 2013). This reaction generates PPi, which is converted into phosphate by a soluble pyrophosphatase (Diaz-Troya et al., 2014). The glycogen synthase (GlgA), later transfers the glucose moiety of the ADP-glucose to the non-reducing end of a linear α-1, 4 glucan. At the end, the glycogen branching enzyme (GlgB) adds α-1, 6 glycosidic bonds synthesizing glycogen branches (Diaz-Troya et al., 2014; Suzuki et al., 2010; Xu et al., 2013). As the nitrogen limitation persists, stored glycogen is oxidized by glycogen catabolic enzymes such as glycogen phosphorylase (encoded by *glgP*) or isoamylases (encoded by *glgX*) (Osanai et al., 2007). Therefore higher expression levels of *glgP* may suggest higher glycogen catabolic activity. As a result, more glucose-1P is converted back into glucose-6P by pgm which is then further catabolized via glycolysis to pyruvate and then through acetyl-CoA to PHB (Osanai et al., 2005). The upregulation of exopolyphosphatases (*ppx*) in the MT_a24 compared to the wild-type PCC 6714 may indicate a higher activity of the enzyme to produce phosphorus from polyphosphate (PolyP). PolyP is synthesized in bacteria from ATP by polyphosphate kinase 1 (PPK1, encoded by *ppk*) and is degraded by exopolyphosphatases (*ppx*), a recessive enzyme which releases the terminal P_i from long-chain linear PolyP (Akiyama et al., 1993). During growth phase and the 4 days of limitation of phosphate, MT_a24 shows upregulation of *ppk* (data not shown) which in turn suggests higher PolyP synthesis activity. As the limitation persists, 9 days of nitrogen and phosphorus limitation, *ppx* is upregulated. Meaning that PolyP is being degraded to

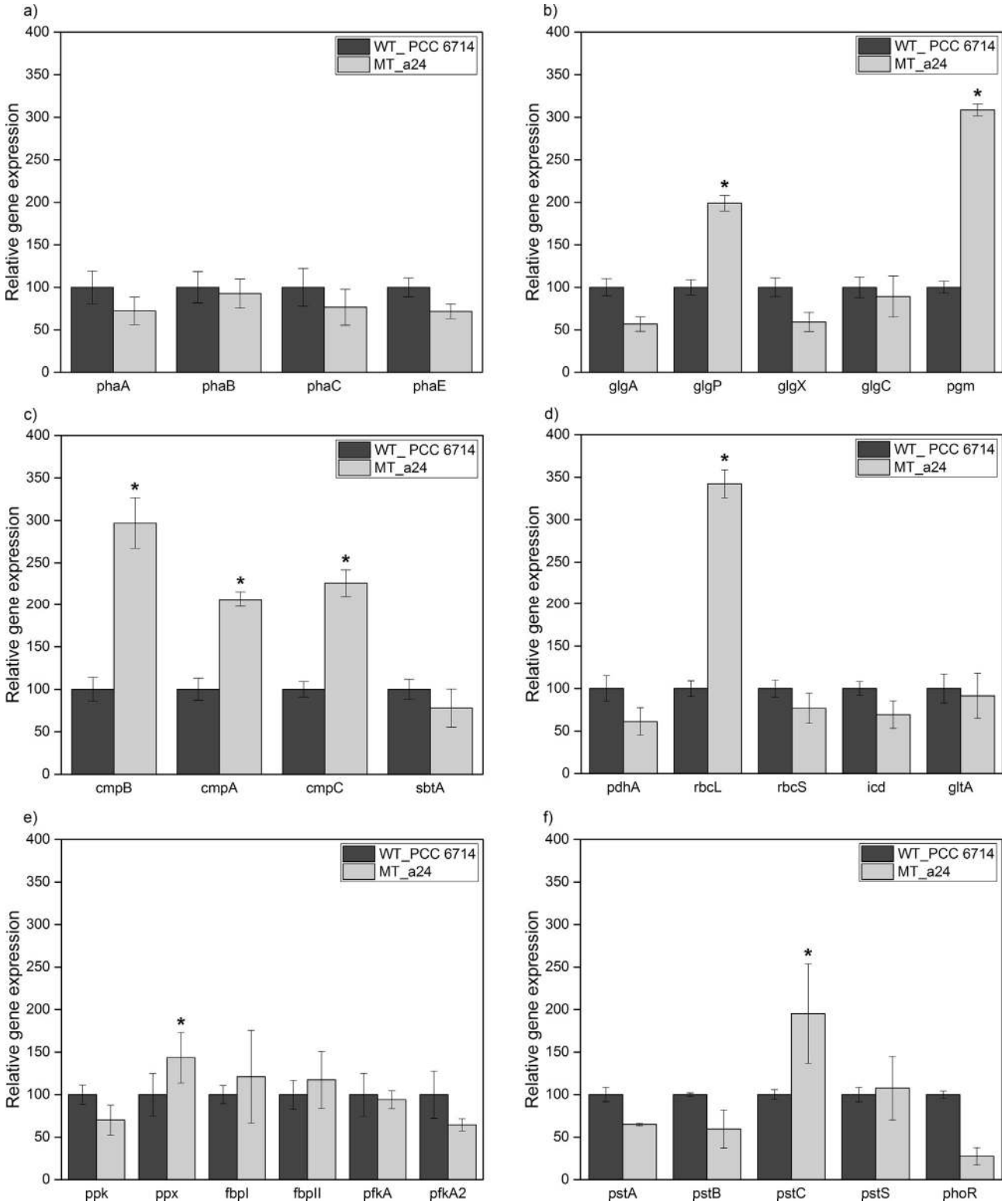

Fig. 6. Quantitative real-time PCR analysis of the wild-type and mutant MT_a24 strain. The relative expression levels of genes involved in (a) PHA biosynthesis (*phaA*, *phaB*, *phaC*, *phaE*), (b) glycogen metabolism (*glgA*, *glgP*, *glgX*, *glgC*, *pgm*) (c) bicarbonate transportation system (*cmpA*, *cmpB*, *cmpC* and *sbtA*) (d) Rubisco (*rbcL* and *rbcS*), TCA cycle (*pdhA*, *gltA* and *icd*) (e) polyphosphate metabolism (*ppk*, *ppx*, *fbpI*,*fbpII*, *pfkA* and *pfkA2*) and (f) phosphate transport system (*pstA*, *pstC*, *pstB*, *pstS* and *phoR*) are shown. Data represent mean ± SD from three independent observations. Statistical analysis was performed using two tailed Student T-test whereas p values < 0.05 were considered statistically significant shown with *. Levels were calibrated relative to that of the wild-type strain *Synechocystis* sp. PCC 6714 under nitrogen and phosphorus limiting conditions (set as 100%).

provide P_i for metabolic and cellular activities, e.g. as a precursor of ATP synthesis.

The evaluation of expression levels of genes responsible for inorganic phosphate transportation indicates upregulation of *pstC* and downregulation of *pstA* and *pstB*. The phosphate regulon sensor histidine kinase (PhoR) which is involved in the phosphorylation of PhoB in

response to environmental signals (Vuppada et al., 2018) was also shown to be downregulated by three-fold. This result along with the previous information obtained from genome sequencing may indicate that the mutation has resulted in a lower activity of the PstA transport channel resulting in a potentially higher activity of PstC indicated by upregulation of *pstC*. Yet not every data obtained could successfully link the genotypes observed for MT_a24 to the superior phenotypes. We hypothesize that maybe imbalanced phosphate transportation is the cause of change in metabolism of MT_a24. One can confirm this by inserting the Pst genes of the MT_a24 into the wild-type strain *Synechocystis* sp. PCC 6714. It has been shown by Monds et al. (2001) that mutations of *pstC* and *pstA* genes of the phosphate-specific transport operon can cause severe phenotypic changes in organisms such as loss of the ability to form biofilms by *Pseudomonas aureofaciens* PA147-2. This report along with our observations propose a more detailed investigation as well as identification of the functional role of genes involved in phosphate transportation in cyanobacteria.

The main goal of this current study was to increase the PHB productivity by random mutagenesis in cyanobacteria to make photosynthetic PHB production competitive with conventional fossil-based polymers. The mutant MT_a24 generated by UV mutagenesis in this study showed more than 2.5-fold higher PHB content under nitrogen and phosphorus limitation compared to that of the wild-type strain *Synechocystis* sp. PCC 6714 from CO_2. Besides the increase in PHB content, superior biomass productivity, higher specific growth rates, and better fitness was also obtained for MT_a24. Therefore, MT_a24 should be considered as a potential strain for PHB production, since the increased PHB productivity brings economical sensibility to cyanobacterial biopolymer production.

4. Conclusions

This study resulted in the characterization of the mutation in the genome of the MT_a24 strain of *Synechocystis* sp. PCC 6714, however, there is much more to explore in this interesting improved cyanobacterium. It would be of great interest to study the influence of phosphate on metabolic pathways and explore its actual role in PHB biosynthesis of cyanobacteria in the future. The results from this work provide clear evidence that the random mutagenesis approach can help identify target genes for future genetic engineering in cyanobacteria.

5. Declaration of conflict of interest

None.

Acknowledgments

The authors would like to thank Christoph Slouka and Alexandra Hofer for critical reading of the manuscript and preparation of the art work presented.

Authors contributions

DK and SP planned the experiments for mutant generation, selection, and characterization and the bioreactor cultivations. DK performed the experiments, analyzed the data and wrote this manuscript. TK and SP designed the primers and planned qPCR experiments. TK and DK performed the quantitative PCR experiments and the data analysis. SP analyzed the data from genome sequencing. CH, ML, ID were responsible for initiation and supervision of the study. ID performed the data analysis for phenotyping microarray experiments. PK wrote a MATLAB script for determination of the CO_2 uptake rates. All authors read and approved the final manuscript.

Funding

Financial support was provided by the “Vienna Business Agency”, [ID 1890571, 2017]. Irina Druzhinina was supported by Austrian Science Fund FWF, P25745-B20.

Availability of data and materials

The data sets supporting the conclusions of this article are included in the main article as well as the supplementary data. The raw data would remain confidential and will not be shared due to a filed patent application (No. A68/2018).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.06.057.

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Appendix

Table S1. Primes for the quantitative PCR. The table represents the forward and backward sequence and accession number for the primers used in quantitative PCR experiment.

Protein	Gene	NCBI Accession number in the reference genome of *Synechocystis* sp. PCC 6714 (Kopf et al., 2014)	Forward 5' – 3'	Reverse
Acetyl-CoA acetyltranferase	*phaA*	D082_14410	CCCGGTGGGAAAGGTAAAAA	AATACAGTGGACGGTTACGC
Acetyl-CoA reductase	*phaB*	D082_14400	TGTGTCAATGAAACCCGGAG	GCGGTACTCAATGTCAACCT
ATP-dependent 6-phosphofructokinase 1	*pfkA*	D082_16980	ATGGGCACAAAACGTATC	TTACATTTCCCCCAAATAGATG
ATP-dependent 6-phosphofructokinase 2	*pfkA2*	D082_24860	ATGGGGGAAATTAAACGC	TTAATCGTTGCCGAGGC
Bicarbonate transport system permease protein	*cmpB*	D082_08860	CCAATACCGACAATGCCAGA	TAATGTGCGTAAGGTCCTGC
Bicarbonate transporter, bicarbonate binding protein	*cmpA*	D082_08870	AGATAGGGCATGGGCATTTG	GCTCGGGATAACGTGGAAAT
Bicarbonate transporter, bicarbonate binding protein 2	*cmpC*	D082_08850	GGGGATAACTGGGATTGCTG	AAATTTTGGAGCGGGTCTGT
Citrate synthase	*gltA*	D082_32700	AACTGCTCCGCTAAATCCTG	CCACTCTGACGGATCCCTAT
Cytochrome b6-f complex subunit	*petA*	D082_26420	CGAGGAACTTAATCCGGGTG	CGAGGAACTTAATCCGGGTG
Exopolyphosphatase	*ppx*	D082_15140	ATGGCTCCTTCCCCTG	CTAACCCTGACTGGATCTG
Fructose-1,6- bisphosphate class 1	*fbpI*	D082_10870	GTGGATAGCACCCTCG	TTAATGCAGTTGGATCACTTTG
Fructose-1,6- bisphosphate class 2	*fbpII*	D082_32800	ATGACCGTTAGCGAG	CTAACTACTTTGCCGTTGTTTG
Glycogene synthase	*glgA*	D082_29730	TTTTCGACCGGGATTACGAC	CTTCCACATACTTGAGGCCC
Glycogen phosphorylase	*glgP*	D082_00830	GCTAGATAGGCCATACGCAC	AATGGTCATTGCCCCTGTTT
Glycogen debranching enzyme	*glgX*	D082_11150	GTTTCTGGATGGTAACGGCT	CCTTCCTCTGGGGTAAAGGA
Glucose-1-phosphate adenylyltransferase	*glgC*	D082_11490	TTTTTCCGAGAAACCCCAGG	GGCCGAGTCAGGAATGATTT
Glycogene synthase	*glgA*	D082_29730	TTTTCGACCGGGATTACGAC	CTTCCACATACTTGAGGCCC
Glycogen phosphorylase	*glgP*	D082_00830	GCTAGATAGGCCATACGCAC	AATGGTCATTGCCCCTGTTT
Glycogen debranching enzyme	*glgX*	D082_11150	GTTTCTGGATGGTAACGGCT	CCTTCCTCTGGGGTAAAGGA
Glucose-1-phosphate adenylyltransferase	*glgC*	D082_11490	TTTTTCCGAGAAACCCCAGG	GGCCGAGTCAGGAATGATTT
Isocitrate dehydrogenase	*icd*	D082_01380	AAAACCTGCTCCACCTCTTG	ATTGGCTACGACGGAGTTTC
PHA polymerase	*phaE*	D082_28620	ATAGCCCTGGAGATCTGCTT	TGGTTAGAGTCCAGCAGTCA
Polyhydroxyalkanoic acid synthase	*phaC*	D082_28610	TCACTGTCTTTCTTGGAGCC	GACCATGCCCATTTTGAACC
Phosphoglucomutase	*pgm*	D082_30550	ATGGCAAGCAGAATTCATC	TTAACCTAGGGCTGAGG
Putative sodium-dependent bicarbonate transporter	*sbtA*	D082_29370	GGGCAGTTAGTGGTTCTACG	ACGGGTTGCTTGCTTAAAGA
Pyruvate dehydrogenase	*pdhA*	D082_14270	CTTTGAGGACAAATGCGCTG	CTACAGCCGGTTTCCTTACC
Polyphosphate kinase	*ppk*	D082_09540	ATGATCCAAGCTATGGCC	TCAAATAATTTCCGCTTCCTCC
Phosphate transport system permease protein	*PstA*	D082_01480	ATGCAACCCCCTAGC	CTAGACATTCCGACGAATG
Phosphate import ATP-binding protein	*PstB*	D082_23250	ATGGCTAATTTACATATTCCCATG	CTAGACATTCCGACGAATG
Phosphate transport system permease protein	*PstC*	D082_23280	ATGGTGGAAGGTTTTTCG	TTAATTGCTCTCCCCTTGG
Phosphate binding protein	*PstS*	D082_23290	ATGCTTAGTTCACTTCAAAAAG	TTATTTGACCTTATCAACCGC
Phosphate regulon sensor protein	*phoR*	D082_25580	ATGGAAATAATTACTTTGGCGATC	CTAGACGTTTTCAGTCTTCG
Protein subunit of Ribonuclease P	*rnpA*	D082_01070	CGCTTTGGCATCACCGTTAG	AGGTGGTTTCTCGAATGCCC
Ribulose bisphosphate carboxylase large chain	*rbcL*	D082_33200	CTTGCCTACAGTTTGGTGGT	GCCTCGAACTCGAACTTGAT
Ribulose bisphosphate carboxylase small chain	*rbcS*	D082_33220	CTTACCTGCCCCCTTTAACC	TCGGCTAGAACTTCATTGGC
Small Subunit Ribosomal RNA	*ssurRNA*	D082_r01	GTGGGGAATTTTCCGCAATG	ATTCCGGATAACGCTTGCAT

Fig S1. The amino acid sequence of the PstA**.** The figure shows the amino acid sequence for the PstA of the published sequence of *Synechocystis* sp. PCC 6714 by Kopf et al. in 2014, the wild-type *Synechocystis* sp. PCC 6714 used in this work and the mutant MT_a24. The amino acid change from alanine to proline in the MT_a24 is located at position 218.

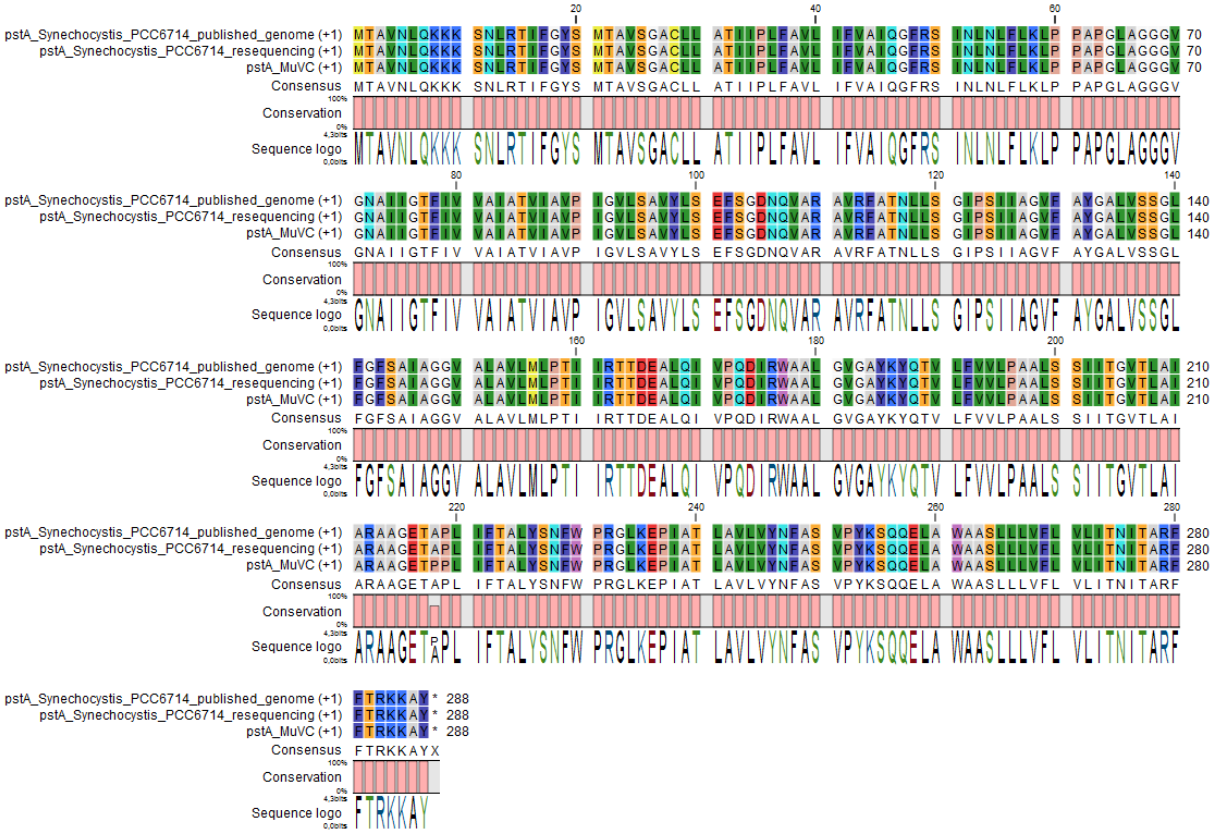

Fig S2. The nucleotide sequence for the *PstA* gene of MT_a24. The nucleotide sequence for the *PstA* of the UV-mutated strain MT_a24 is shown. The nucleotide change in the MT_a24 is located at position 652 and is indicated in red.

ATGACCGCCGTTAACCTCCAAAAGAAAAAATCTAACCTGAGAACCATCTTTGGTTACTCTA
TGACTGCTGTGTCGGGGGCCTGTTTATTGGCCACCATTATTCCCCTGTTTGCGGTGCTAAT
TTTTGTTGCCATCCAGGGTTTCCGCAGCATTAACCTTAACCTATTTCTCAAACTACCCCCGG
CCCCGGGTTTGGCTGGTGGTGGAGTGGGCAATGCCATTATCGGAACCTTTATCGTGGTGG
CGATCGCCACGGTGATTGCTGTGCCCATTGGAGTTTTGAGTGCGGTTTATCTTTCCGAGTT
TAGCGGTGATAACCAAGTGGCCCGGGCAGTACGTTTTGCCACCAACCTGTTGAGTGGCAT
TCCTTCCATCATTGCGGGGGTATTTGCCTATGGGGCGTTGGTGTCTTCGGGACTATTTGGC
TTTTCTGCCATTGCGGGGGGCGTTGCCCTGGCGGTGTTGATGTTGCCCACCATTATCCGTA
CCACCGACGAAGCGTTACAGATTGTGCCCCAGGATATTCGTTGGGCAGCTCTGGGGGTGG
GAGCTTACAAATACCAGACCGTTTTATTCGTCGTTCTGCCCGCTGCCCTTTCTTCCATTATC
ACCGGGGTCACATTGGCGATCGCCAGGGCTGCTGGAGAAACG**C**CCCCCCTAATTTTCAC
TGCCTTATATTCCAACTTCTGGCCCCGGGGACTGAAAGAACCCATCGCGACACTGGCTGTA
TTGGTTTATAACTTTGCCTCGGTACCCTACAAATCTCAGCAAGAATTGGCCTGGGCAGCTT
CGTTGTTGCTGGTGTTTTTGGTGCTCATTACCAACATCACCGCTCGCTTCTTTACTCGGAAG
AAGGCTTATTAG

5.3 Bioprocess optimization and understanding of physiological responses

5.3.1 Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: bioprocess understanding and evaluation of productivities

Abstract: Recently, several mutants of *Synechocystis* sp. PCC 6714 were obtained showing superior PHB content and productivities. Here, the most promising mutant named MT_a24 is compared in detail with the wild-type in controlled photobioreactors. In order to provide an easily scalable and alternative approach to the normally done two-step process -comprising of growth phase and limitation phase- a one-step cultivation was optimized. The multivariate experimental design approach was used for the optimization of the one-step, self-limiting media. During one-step cultivation of MT_a24 with optimized media $30 \pm 4\%$ (DCW) corresponding to 1.16 g L^{-1}, PHB was obtained. Using pulse experiments it was demonstrated that phosphate is the key driver of glycogen synthesis in *Synechocystis* sp. PCC 6714 and it can be used to boost glycogen productivity. The maximum glycogen content acquired was 2.6 g L^{-1} (76.2 % DCW) for mutant MT_a24 using phosphate feeding and carbon dioxide as carbon source.

This manuscript is published in the Journal of Bioresource Technology.

Kamravamanesh Donya, Slouka Christoph, Limbeck Andreas, Lackner Maximilian, Herwig Christoph. Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: bioprocess understanding and evaluation of productivities. Bioresource Technology 273 (2019) 277-287.

Bioresource Technology 273 (2019) 277–287

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: Bioprocess understanding and evaluation of productivities

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ARTICLE INFO

Keywords:
Synechocystis sp. PCC 6714
Glycogen
Poly-β-hydroxybutyrate
Phosphorus
Media optimization
Carbon dioxide

ABSTRACT

Recently, several mutants of *Synechocystis* sp. PCC 6714 were obtained showing superior PHB content and productivities. Here, the most promising mutant named MT_a24 is compared in detail with the wild-type in controlled photobioreactors. In order to provide an easily scalable and alternative approach to the normally done two-step process -comprising of growth phase and limitation phase- a one-step cultivation was optimized. The multivariate experimental design approach was used for the optimization of the one-step, self-limiting media. During one-step cultivation of MT_a24 with optimized media 30 ± 4% (DCW) corresponding to 1.16 g L^{-1} PHB was obtained. Using pulse experiments it was demonstrated that phosphate is the key driver of glycogen synthesis in *Synechocystis* sp. PCC 6714 and it can be used to boost glycogen productivity. The maximum glycogen content acquired was 2.6 g L^{-1} (76.2% DCW) for mutant MT_a24 using phosphate feeding and carbon dioxide as carbon source.

1. Introduction

Biodegradable plastics, due to their eco-friendly nature offer a viable solution to environmental problems caused by fossil-based plastics (Getachew and Woldesenbet, 2016). Poly-β-hydroxybutyrate (PHB) is the most promising and one of the only 100% biodegradable polymer when compared to other bioplastics (Getachew and Woldesenbet, 2016; Hrabak, 1992). It shows similarity in thermal properties and tensile strength to those of polyesters (Verlinden et al., 2007) and therefore can replace them in many applications such as food service and product packaging (Tan et al., 2014). Also, PHB can replace the commodity polymer polypropylene (PP) in a variety of applications (Haenggi, 2013; Markl et al., 2018; Yeo et al., 2018). Under stressed growth conditions, such as nitrogen and phosphorus limitations, bacteria and some archaea synthesize PHB as storage material using sugar and organic compounds as the carbon source (Galia, 2010). High production cost in comparison to fossil-based plastics is one major bottleneck for the commercialization of PHB (JB and Yves, 2008). The costs for PHB production are significantly above those for fossil commodity polymers and other bioplastics materials such as thermoplastic starch (TPS), polybutylene adipate terephthalate (PBAT) or polylactic acid (PLA) (Lackner, 2015). Lately, attention has been focused to reduce the production cost of PHB using cyanobacteria as production host and sustainable resources. Cyanobacteria use CO_2 as the main carbon source as they are capable of photosynthesis (Carpine et al., 2017). They are considered as cell-factories with the remarkable metabolic flexibility to adapt and to thrive in versatile environments (Pikuta et al., 2007; Singh, 2009). However, from an economic point of view, photosynthetic PHB production in cyanobacteria has a major disadvantage, as the native PHB content is normally very low (Drosg, 2015) – less than 10% dry cell weight (DCW). In order to commercialize the cyanobacterial PHB and therefore compete with fossil-based conventional

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https://doi.org/10.1016/j.biortech.2018.11.025
Received 18 September 2018; Received in revised form 6 November 2018; Accepted 7 November 2018
Available online 09 November 2018
0960-8524/

plastics and cornstarch/sugar-based bioplastics in the marketplace, production of photosynthetic PHB needs significant improvement. Enhancement of PHB productivity in cyanobacteria using various methods such as genetic modification has shown little success. The authors have previously shown an increase in PHB productivity of up to 37 ± 4% (DCW) under nitrogen and phosphorus limitation for mutant MT_a24, a randomly mutated strain of *Synechocystis* sp. PCC 6714 (Kamravamanesh et al., 2018). During genome sequencing the high PHB producing mutant MT_a24 (Kamravamanesh et al., 2018) showed a mutation in a phosphate transport system, PstA, having a strong impact on the available phosphate content inside the cell. While the mutant MT_a24 showed a significantly higher PHB productivity the subsequent goal is the understanding of the supremacy of the mutation and therefore using the knowledge for improvement and stabilization of the production process.

As with other bacteria, cyanobacteria have four different phases of growth: lag phase, exponential (or log) phase, stationary phase and the death phase (Li et al., 2014; Wood et al., 2005). PHB production in cyanobacteria mainly occurs during the stationary phase or the death phase. In order to maintain high PHB content in a continuous mode of operation and to minimize the biomass loss, optimization of culture media seems compelling. The most important nutrient parameters are macronutrients nitrogen (N) and phosphorus (P) (Li et al., 2014). Nitrogen is required for the synthesis of proteins, genetic material, and other cell structures. Inorganic phosphate (Pi) is an essential compound for cyanobacterial energy metabolism and also a component of nucleic acids, phospholipids and other cell constituents (Vuppada et al., 2018). Cyanobacteria accumulate PHB under limiting growth conditions, making, therefore, the PHB production in a two-step process comprising of a growth phase followed by a nutrient limitation phase. It has been known that during nitrogen and phosphorus limitation, cyanobacteria redirect carbon to internal stores to maintain a basal growth rate and to partition excess carbon (Gorl et al., 1998), leading to an up-regulation of sugar catabolic pathways and accumulation of carbon reserve polymers (Purcell-Meyerink et al., 2016) such as glycogen and PHB.

Glycogen in cyanobacteria shows a double function: one as a carbon reserve and also as a buffering substance. Recently photosynthetic production of glycogen has gained a great importance as it can be converted to value-added compounds such as bioethanol using yeast fermentation (Aikawa et al., 2013; Ho et al., 2013). However, the impact on the increase in glycogen has not been discussed yet. In case of heterotrophic bacteria it has been shown that the intracellularly stored polyphosphate and glycogen produce the energy and reducing the power required for the storage of volatile fatty acids such as PHB (Lopez-Vazquez et al., 2009; Mino et al., 1998).

In cyanobacteria, the acetyl-CoA required for PHB synthesis can be obtained via two main routes, i) direct carbon dioxide fixation via the Calvin-Benson cycle and ii) via the glycogen oxidation by glycogen catabolism (Gao et al., 2012) using glycogen phosphorylase as the key enzyme (Diaz-Lobo et al., 2015). The acetyl-CoA is converted to acetoacetyl-CoA by β-ketothiolase (encoded by *PhaA*) (Taroncher-Oldenburg et al., 2000). This acetoacetyl-CoA is then reduced by acetoacetyl-CoA reductase (encoded by *PhaB*) to 3-hydroxybutyryl-CoA (3HB) (Taroncher-Oldenburg et al., 2000) and at the end, the PHB synthase, encoded by phaE and phaC genes, catalyzes the polymerization of 3HB to PHB (Hein et al., 1998). The fundamental challenge of PHB or glycogen production from cyanobacteria lies in the ability to manipulate cellular partitioning of macromolecules to enhance the ratio of desired product-to-biomass (Purcell-Meyerink et al., 2016).

In this current study, the authors also aim to establish an easily scalable and controllable bioprocess for photosynthetic production of PHB. For this purpose, a self-limiting one-step process was optimized in order to replace the commonly done two-step processes. To achieve this goal, media optimization was done using the multivariate experimental design (DoE) approach. Also, the significance of nitrogen and phosphorus on biomass growth and PHB production during a one-step process was revealed. Consequently, a one-step cultivation strategy was developed with the optimum dosage of macronutrients such as nitrogen and phosphorus using cyanobacterial mutant strain MT_a24.

Further the mutant MT_a24 and the wild-type strain *Synechocystis* sp. PCC 6714 were cultivated using various operation modes and were compared in terms of stoichiometry and performance under various cultivation strategies. Also, the raw data obtained from various cultivations was underpinned with a detailed quantitative physiological analysis, as per our knowledge, so far not reported for cyanobacteria in such a depth.

Further, the impact of phosphorus during PHB and glycogen biosynthesis was assessed using various cultivation strategies, such as pulse experiments. The mutant MT_a24 was used to investigate carbon partitioning and to enhance the ratio of glycogen-to-biomass production using a tuned feeding strategy.

2. Material and methods

2.1. Strain and inoculum preparation

An axenic culture of wild-type strain *Synechocystis* sp. PCC 6714 was purchased from Pasteur Culture Collection of Cyanobacteria (Pasteur Institute, Paris, France). Unless stated otherwise, *Synechocystis* sp. PCC 6714 was grown in BG-11 medium (Rippka et al., 1979) supplemented with 10 mM HEPES buffer pH 8.2 with the addition of 15 g L^{-1} of Kobe agar for plates and 5 mM $NaHCO_3$ as carbon source prior to inoculation. In order to induce nitrogen deficiency, cells were cultured in BG-11 media without nitrate and ammonia. The $(NH_4)_5[Fe(C_6H_4O_7)_2]$ and $Co(NO_3)_2{\cdot}6H_2O$ were substituted with equimolar concentrations of $FeC_6H_5O_7$ and $CoCl_2{\cdot}6H_2O$ in terms of iron and cobalt content. For phosphorus limitation, KH_2PO_4 was replaced with an equimolar concentration of KCl in terms of potassium content.

2.2. Design of experiments (DoE)

Full factorial experimental design with three center points and data evaluation were carried out using MODDE (Umetrics, Sweden). For each selected response, MODDE generates a multiple linear regression (MLR) model with key parameters of R^2 (coefficient of determination), Q^2 (predictability), RP (reproducibility) and MV (model validity). The thresholds for R^2, Q^2, and RP were 0.5 for each parameter. Models with an MV value of > 0.25 were considered to be significant.

2.3. Growth determination and estimation of dry weight

Biomass growth was determined spectrophotometrically at 750 nm using a UV–Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 24-h intervals. Dry cell weight (DCW) was determined in triplicates by transferring 5 mL of fermentation broth into pre-weighed glass tubing and centrifuged at 8000 rpm for 10 min at 4 °C. The pellets were dried overnight at 85 °C and dry weight was determined gravimetrically. A correlation between optical density and DCW could be established, taking into account that this correlation changes over cultivation time and is described in Eq. (1).

$$C_x = 0.37 \times OD_{750}. \quad [g\ L^{-1}] \tag{1}$$

2.4. Bioreactor cultivations

Bioreactor experiments were carried out under sterile conditions as previously described (Kamravamanesh et al., 2018) in a DASbox Mini Bioreactor System (Eppendorf AG, Hamburg, Germany) with a maximum working volume of 250 mL. The scale-up was done in a 1.5 L jacketed, glass-reactor with a working volume of 1 L (Applikon B.V, the Netherlands). The pH was maintained at 8.5 by addition of 0.5 M HCl or NaOH added to the reactors with a DASGIP MP, multi-pump module

(Eppendorf AG, Germany). The agitation was set at 300 rpm and the reactors were bubbled with a mixture of sterile filtered air and 2% CO_2 at a flow rate of 0.4 vvm (6 L h^{-1}). The illumination was done using LED strips wrapped uniformly around the reactor vessels providing a light intensity of 50 µmol m^{-2} s^{-1} photons in PAR (Wood et al., 2005). pH and dissolved oxygen were measured using, Visiferm DO sensor (Hamilton, Reno, NV, USA) and EasyFerm plus PHI K8 sensor (Hamilton, Switzerland). Online biomass was determined by an OD sensor (Eppendorf DASGIP OD4 Module, 880 nm). The exhausted gas was analyzed by a DASGIP GA4 gas sensor module (Eppendorf AG, Hamburg, Germany) with a ZrO_2 sensor for O_2 and infrared CO_2 sensor technology. All fermentation parameters and variable pump set-points were controlled using the DASware control system (Eppendorf AG, Hamburg, Germany).

2.4.1. One-step, two-step, and three-step cultivation

In this study, the terms one, two or three-step process are used referring to the mode of operation. The one-step cultivations were done using an optimized self-limiting media, where the growth and subsequent limitation occurred using a single media.

For two-step cultivations, in order to maximize biomass concentration, the cells were allowed to grow on BG-11 complete media with sufficient amount of nutrients for 5–6 days. To induce limitation, cells were harvested under sterile conditions by centrifugation at 4000 rpm at room temperature and were re-suspended in media without N and P source.

Three-step cultivations were done to obtain sequential limitations of nutrients. The cultivations were done by allowing the cells to grow on BG-11 media with elevated concentration of phosphorus for 5–6 days. For the second step, cells were harvested under sterile conditions by centrifugation at 4000 rpm at room temperature and were re-suspended in BG-11 media without nitrogen source. The third step was done as the second step only by transferring the cells into BG-11 media without nitrogen and phosphorus source.

Photobioreactor experiments were performed three-times and one cultivation has been shown here as an example. Also for the mutant MT_a24 experiments were performed every time in biological duplicates in order to check the stability of the mutation. Samples were taken in triplicates at 24-h intervals and were analyzed for dry cell weight, glycogen, and PHB content.

2.5. Determination of glycogen content

Glycogen quantification was performed using a protocol from Forchhammer and Tandeau de Marsac (1995) with slight modifications. Pre-weighed dried cells from 2 mL culture were heated with 1 mL of 7.5% v/v of the sulfuric acid solution at 95 °C on a heating block (Accublock™, Labnet, USA) for 120 min. Glucose was produced from glycogen by acid hydrolysis, the hydrolysate was then quantified by ion-chromatography using the method by Hofer et al. (2018). For calibration, pure glycogen (Sigma- Aldrich, USA) was treated accordingly and analyzed in parallel with samples. The method was run on an Ion chromatography ICS-5000 (ThermoFisher Scientific, USA), equipped with a pump (LPG), an autosampler (AS-AP) with a 25 µL sample loop, a detector compartment (DC) and an electrochemical detector. Chromeleon 7.2 was used for the control of the devices as well as for the quantification of the peak areas. The glycogen content per (DCW) was calculated as explained in Eq. (2).

$$\%\text{DCW } Gly = \frac{\text{mg (Gly)}}{mg\ (DCW)} * 100. \quad (2)$$

2.6. Determination of the PHB content

PHB quantification was done using the procedure described by Schlebusch and Forchhammer (2010). Pre-weighed dried cells (2–5 mg) were boiled with 1 mL conc. H_2SO_4 at 100 °C on a heating block (Accublock™, Labnet, USA) for one hour to convert PHB to crotonic acid. Samples were diluted 20 times using 0.014 M H_2SO_4. Crotonic acid was determined using a high-performance liquid chromatography system (Thermo-Fischer Scientific, USA) with a Nucleosil C8 column (Macherey-Nagel, Germany) using an isocratic elution method. The mobile phase used was 20 mM NaH_2PO_4 buffer; pH 2.5 and acetonitrile (70:30 v/v) with a flow rate of 0.85 mL min^{-1} and a column temperature of 30 °C. Detection of crotonic acid was done using a diode array detector (DAD) detector (Thermo-Fischer Scientific, USA) at 210 nm. For calibration, pure PHB (Sigma- Aldrich, USA) was treated accordingly and analyzed in parallel with samples. Instrument control and peak evaluation were done with Chromeleon 7.2 (Thermo-Fischer Scientific, USA). The percentage PHB per (DCW) was determined according to Eq. (3):

$$\%\text{DCW } PHB = \frac{\text{mg (PHB)}}{mg\ (DCW)} * 100. \quad (3)$$

2.7. Analysis of macronutrients

The concentrations of nitrogen (N), phosphorus (P), sulfur (S), and carbon (C) were measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Prior to analysis samples were diluted (1:2) with 1% (v/v) hydrochloric acid and aluminum was added as an internal standard with a final concentration of 5 µg mL^{-1}. Stock solutions for P, N, S and C were prepared from analytical grade salts of sodium dihydrogen phosphate dihydrate, potassium nitrate, sodium sulfate and sodium acetate. Standard solutions required for external calibration were prepared just before use by appropriate dilution of the stock solution with 1% (v/v) hydrochloric acid; followed by the addition of the internal standard.

Samples and standards were analyzed with an iCAP 6500 ICP-OES spectrometer (Thermo Scientific, USA) equipped with a conventional Meinhard-type glass nebulizer and a quartz cyclonic spray chamber. Sample-uptake was performed in the self-aspirating mode of the nebulizer, and the peristaltic pump of the instrument was used to drain the spray-chamber only. Background-corrected emission signals were recorded in the radial viewing mode and processed using Qtegra software (Thermo Scientific, USA). Three replicates with an integration time of 10 s each were measured for samples as well as standard solutions. The optimized ICP parameters are summarized in Table 1. Observed signal intensities were normalized using the signal response for the internal standard (Al) and finally converted into concentration units by means of external aqueous calibration. Derived Al signals were constant over each measurement session (less than 5% relative standard deviation for the whole measurement period, indicating the absence of temporal trends), and no significant difference in Al-response between samples and calibration standards was observed.

Table 1
Represents the optimized ICP parameters used for determination of nitrogen, phosphorus, sulfur, and carbon.

RF power	1400 W
nebulizer gas flow	0.8 L min^{-1} argon
cooling gas flow	15 L min^{-1} argon
auxiliary gas flow	2 L min^{-1} argon
viewing height above load-coil	9 mm
N 174.272 nm	*
P 213.618 nm	P 178.284 nm
S 182.034 nm	S 180.731 nm
C 175.183 nm	C 193.091 nm

* N has only one useful emission line in the spectral range investigated.

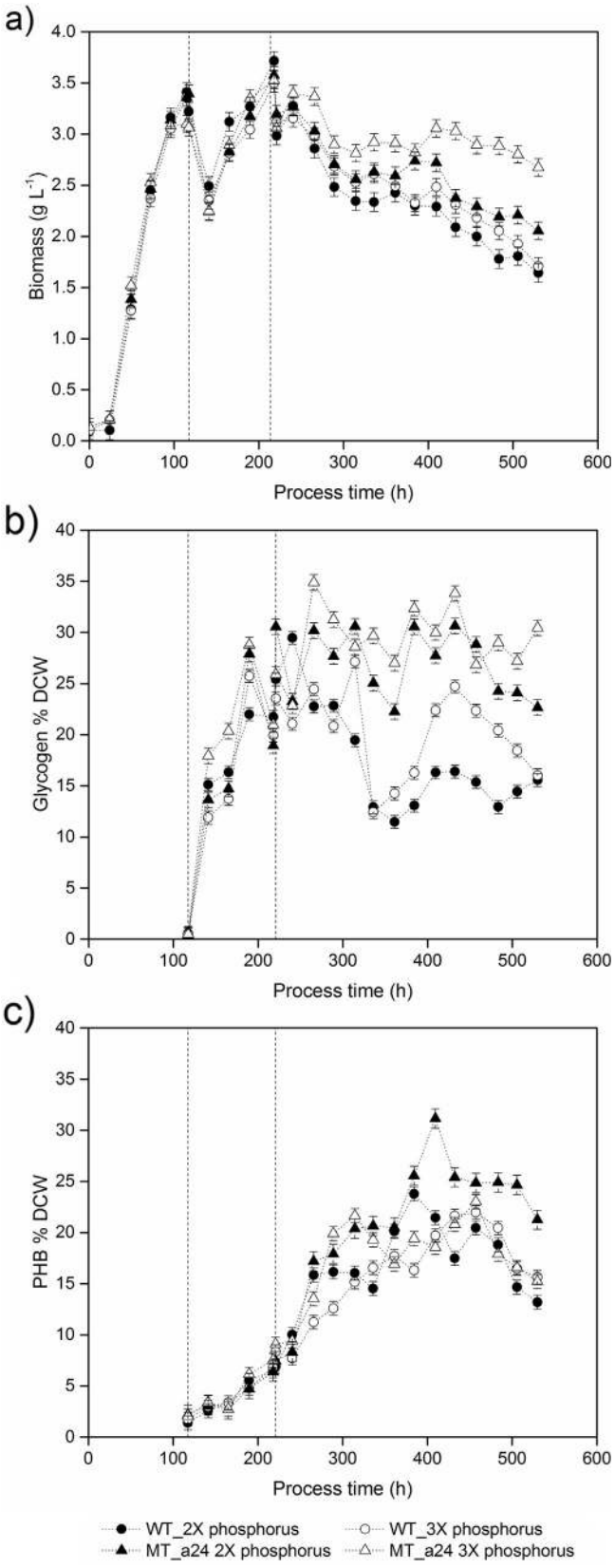
3. Results and discussion

3.1. Three-step cultivation

The authors have previously shown that the cause for the superiority of the mutant MT_a24 over its parent-strain *Synechocystis* sp. PCC 6714 is a mutation in phosphate system transport protein, PstA. Even though the MT_a24 did not show higher intracellular phosphorus content at the measured time-points it was speculated that maybe elevated phosphorus concentration at some stage is driving enzymatic activities towards a particular pathway. In order to address the rising question- in which biosynthetic step does phosphate perform- a step-by-step process was designed where the limitation of nitrogen and phosphorus was done sequentially. The three-step cultivation using WT_PCC 6714 and the mutant MT_a24 was performed in a multi-photobioreactor system with elevated phosphate concentrations during the growth phase. For the first step, complete BG-11 media one with 2× phosphorus concentration (80 mg L^{-1}) and other with 3× phosphorus concentration (120 mg L^{-1}) was used. For the second step BG-11 media without nitrogen source was used. Finally, the third step was done using BG-11 media without nitrogen and phosphorus source.

The Fig. 1 represents biomass growth, glycogen and PHB content for the three-step cultivation of wild-type *Synechocystis* sp. PCC 6714 and mutant MT_a24 with elevated phosphorus content. During the step-one when all media components were abundant no glycogen or PHB was produced (Fig. 1). Surprisingly, the elevated phosphorus concentration was stimulating to biomass production in the wild-type PCC 6714 while it was previously observed that the mutant MT_a24 was superior in terms of biomass growth to the WT_PCC 6714. As shown in Fig. 1. a) both mutant MT_a24 and WT_PCC 6714 obtained more or less similar biomass concentration during growth and nitrogen limitation step (3.5 ± 0.4 g L^{-1}). During the last step of the process where both nitrogen and phosphorus were limited MT_a24 with 3× phosphorus content showed the highest biomass concentration of 3 ± 0.3 g L^{-1}. During the nitrogen limitation step, glycogen was produced as the main carbon reserve in all four cultivation with and PHB content remained around 5% DCW. No significant difference was observed in the maximum glycogen content (more or less 30 ± 2% DCW) during the nitrogen limitation step in WT_PCC 6714 and MT_a24 with different phosphorus concentrations. In contrary to what was expected not only nitrogen limitation but also phosphorus limitation triggered glycogen production. As a result, the glycogen content further increased during the last step (N and P limitation) in all four cultivations (Fig. 1b). For both MT_a24 cultivations, the glycogen content stayed stable to a value of approximately 30 ± 2% (DCW).

For the mutant MT_a24, both higher biomass concentration during nitrogen and phosphorus limitation and higher glycogen content was obtained when more phosphorus was provided. It seems like for mutant MT_a24 higher phosphorus contents lead to the stability of the glycogen production, therefore, not maximizing the PHB content. During the final step -nitrogen and phosphorus limitation step- both mutant and WT_*Synechocystis* 6714 produce PHB as the main carbon reserve. For both the wild-type and MT_a24 higher PHB content was observed when lower phosphate concentration was provided during the first two steps. However, for both strains, the higher glycogen content was obtained when higher phosphate (3× phosphate) was used during the previous steps. The maximum PHB content of 31 ± 3% (DCW) was acquired for MT_a24 with 2× phosphorus concentration followed by the MT_a24 with 3× phosphorus and WT_PCC 6714 with 2× phosphorus concentration with the similar value of 23 ± 2% (DCW), Fig. 1c). The lowest PHB content of 22 ± 2% (DCW) was obtained for the wild-type with 3× phosphorus concentration. The values obtained for the wild-type strain under elevated phosphorus concentration are yet much higher than previously observed for this strain. This could indicate that elevated phosphorus concentration is stimulating to wild-type strain PCC 6714 by increasing the biomass growth and also PHB production.



(caption on next page)

The results obtained here might be a clear evidence, proving that higher phosphorus can stimulate growth and the PHB production in *Synechocystis* sp. PCC 6714 indicating that higher availability of intracellular phosphorus content is the reason for the superior performance of the

Fig. 1. Three-step cultivation of wild-type Synechocystis sp. PCC 6714 and mutant MT_a24 with elevated phosphorus concentration. a) The growth curve for the WT_ Synechocystis sp. PCC 6714 and the MT_a24, each with 2× (80 mg L^{-1}) and 3× (120 mg L^{-1}) phosphorus content. b) Glycogen in % (DCW) for the WT_ Synechocystis sp. PCC 6714 and the MT_a24 each with 2× and 3× phosphorus concentration and c) PHB content in % (DCW) for the three-step cultivation of WT_ Synechocystis sp. PCC 6714 and the MT_a24 using 2× (and 3× phosphate. The vertical line at 120 h represents the start of the second step (N limitation) and the vertical line at 220 h represent the start of third step (N and P limitation). Data represent the mean ± SD of triplicate measurements.

MT_a24.

3.2. Impact of macronutrients for an optimal one-step cultivation

In this study, one of the main objectives was to establish a simple, one-step, and optimized bioprocess for the industrial production of PHB using MT_a24. As the previous screening studies (Kamravamanesh et al., 2017) on the wild-type strain *Synechosystis* sp. PCC 6714 had shown, the maximum PHB content could be obtained when both nitrogen and phosphorus were limited. Here based on the results obtained from the earlier experiment (three-step cultivation, Fig. 1) it was hypothesized that for an ideal one-step cultivation with maximum productivity an optimum starting concentration of phosphorus is required. To that end, the significance of macronutrients (N and P) on biomass growth and PHB production as well as the appropriate dosage during one-step cultivation was determined using multivariate experimental design. The DoE was used to minimize the error in determining the influential parameters, allowing systematic and efficient variation of all factors (Ooijkaas et al., 1999). Table 2 displays the experiment and parameter ranges for a full-factorial design of experiments, the parameter ranges were selected in a manner to induce limitation of nutrients N and P. The two responses, biomass growth, and PHB accumulation was monitored daily and the results of the DoE obtained after 14 days of cultivation were determined and were analyzed with MODDE. In total thirteen experiments were performed in the shake-flasks. Out of which seven test conditions were suggested by MODDE, and six experiments were added to detect the interactive and quadratic terms if any. The experiments were carried out using wild-type *Synechocystis* sp. PCC 6714 and BG-11 media with different nitrogen and phosphorus concentrations, provided in Table 2.

At first, the effect of nitrogen and phosphorus concentration was studied on PHB accumulation. A valid model was obtained for PHB accumulation (MV = 0.77) showing that (Fig. 2a) PHB accumulation was significantly increased by elevated phosphorus concentrations. This effect was not significant for nitrogen concentration (shown in coefficient and observed vs. predicted plots in the Supplementary data). The interaction term nitrogen * phosphorus also showed significance by reducing the PHB content. Within the borders of the experimental matrix, highest PHB accumulation of 21% (DCW) was obtained when both nitrogen and phosphorous were maximum (1 g L^{-1} for $NaNO_3$ and 60 mg L^{-1} for $K_2HPO_4{\cdot}3H_2O$). The obtained contour plot (Fig. 2a) indicates that elevated phosphorus concentration during a one-step cultivation may increase the PHB content.

Next, the effect of initial nitrogen and phosphorus concentration, on biomass formation was determined. As shown in the contour plot, Fig. 2b) and the observed vs. predicted plot (Supplementary data) in order to obtain higher biomass concentration both higher nitrogen and higher phosphorous concentration are required. For biomass concentration, a valid model (MV = 0.53) was obtained showing that both factors, nitrogen, and phosphorus had a significant influence on the final biomass concentration. Also, the interactive term nitrogen * phosphorus showed significance in biomass formation having a negative impact on final biomass content. An indication that in order to obtain both high biomass as well as PHB content both factors need to be considered. Within the borders of the experimental matrix, highest biomass concentration of OD_{750} was found at 1 g L^{-1} nitrate and 60 mg L^{-1} of phosphate after 14 days of cultivation.

Table 2
Experimental design for optimization of nitrogen and phosphorus source. Experimental design, the PHB content in % (DCW) obtained and the biomass in OD_{750} nm are shown for the full-factorial DoE.

Experiment	Nitrate concentration (mg L^{-1})	Phosphate concentration (mg L^{-1})	PHB (% DCW)	Biomass (OD_{750})
N1	100	5.0	8.0	0.7
N2	500	5.0	8.5	1.4
N3	100	20	13.0	1.0
N4	500	20	15.7	1.78
N5	300	12.5	11.2	1.33
N6	300	12.5	11.7	1.41
N7	300	12.5	13.0	1.34
N8	300	5.0	10.8	1.01
N9	100	12.5	11.5	0.8
N10	300	20	16.0	1.43
N11	500	12.5	12.7	1.65
N12	1000	40	19.0	2.0
N13	1000	60	21.0	2.1

3.3. One-step vs. two-step cultivations

A commonly used approach to establishing production process in cyanobacteria is using two-step cultivation strategy, with a growth step where nutrients are abundant followed by a production step where nutrients such as nitrogen or phosphorus are limited. This strategy is difficult to establish for production in large-scale and in most cases, the productivities reduce over cultivation time, therefore, it's of great importance to establish an easy, one-step cultivation showing maximum product content. Previously a high PHB producing mutant of *Synechocystis* sp. PCC 6714, namely MT_a24 was generated using UV-mutagenesis (Kamravamanesh et al., 2018).

From the DoE experiments it could be anticipated that, higher phosphorus concentration during one-step cultivations may be a driving force for the metabolic pathways towards PHB production. PHB production was studied in wild-type strain *Synechocystis* sp. PCC 6714 and the mutant MT_a24 using one-step cultivation with elevated phosphorus content and the values for nitrate and phosphate concentration used here were obtained from the earlier DoE experiment (1 g L^{-1} for $NaNO_3$ and 60 mg L^{-1} for $K_2HPO_4{\cdot}3H_2O$). The cultivations were done in a Dasbox multi-bioreactor system where the one-step and two-step cultivations were performed in parallel.

As indicated in Fig. 3a) and c) both wild-type and mutant MT_a24 show increased PHB content during one-step cultivations with elevated phosphorus concentrations when compared to two-step cultivations (Fig. 3b) and d)). During one-step cultivation the WT_PCC 6714 obtained maximum of 32 ± 4% (DCW) glycogen after 230 h of the process and 20.4 ± 2% (DCW) PHB after 430 h of the cultivation (Fig. 3a). The values obtained for the wild-type during two-step cultivation were 31 ± 4% (DCW) glycogen and 16.4 ± 2% (DCW) PHB (Fig. 3b). For the one-step cultivations of the mutant MT_a24, 61.7 ± 7% (DCW) glycogen was obtained after 190 h of the process and 35 ± 4% (DCW) PHB after 428 h (Fig. 3c). As indicated in Fig. 3 d the MT_a24 showed a maximum of 49.5 ± 5% (DCW) glycogen after 261 h and 30 ± 4% (DCW) PHB after 500 h of a two-step process with nitrogen and phosphorus limitation.

The maximum biomass concentration obtained for the wild-type *Synechocystis* sp. PCC 6714 during one-step cultivation was 3.2 ± 0.3 g L^{-1} which is higher than the value obtained for wild-type during the two-step process (2.9 ± 0.3 g L^{-1}). For the strain MT_a24, the maximum biomass concentration obtained during the one-step process was 3.9 ± 0.4 g L^{-1} which was slightly lower than the

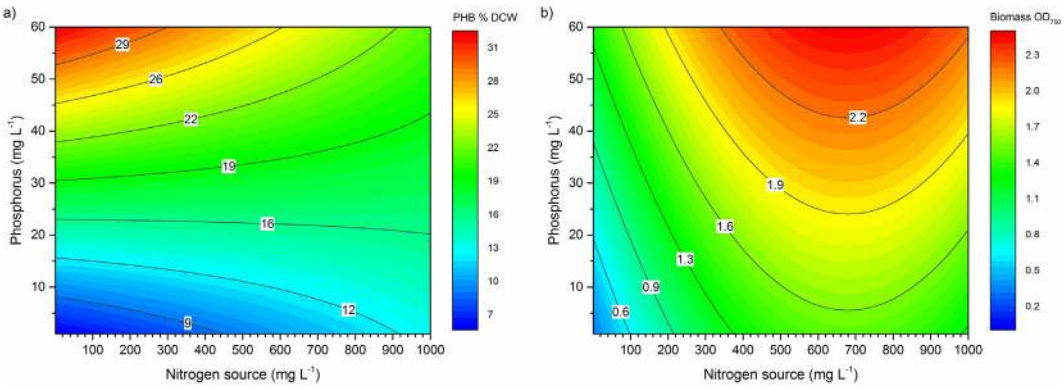


Fig. 2. Contour plot for optimization of biomass growth and PHB production in *Synechocystis* sp. PCC 6714 respective to N and P availability. The contour plot shows the interaction terms of two factors namely initial phosphate concentration and initial nitrate concentration a) for PHB content in % (DCW) and b) for biomass growth measured by optical density at 750 nm (OD_{750}). The sweet spot is indicated in the shade of red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

maximum biomass concentration of 4.3 ± 0.4 g L^{-1} during two-step cultivation.

3.4. Physiological analysis

The PHB production process in *Synechocystis* sp. PCC 6714 can be divided into three essential phases. The phase-I which consists of biomass growth, where nutrients are abundant and the focus is directed towards biomass formation. The phase-II or glycogen production phase, presenting the beginning of nitrogen and phosphorus limitation, where the biomass growth is accompanied by glycogen production and glycogen is synthesized as the major intracellular carbon storage. During the phase-II, PHB is synthesized mainly from CO_2. The phase-III, PHB production phase or the interconversion-phase, during which, glycogen is degraded and partially converted into PHB. Using the measured dry cell weight, glycogen and PHB concentration and the residual nitrogen and phosphorus content over process time, the average volumetric and specific productivities were determined for the mentioned three phases of individual cultivations and were compared for mutant MT_a24 and its parent strain wild-type *Synechocystis* sp. PCC 6714.

The analysis of the data provided in Table 3 indicates that the highest average biomass production rate was obtained during the phase-I, of a three-step cultivation for the MT_a24 with 2× phosphate showing the value of 660 ± 50 mg L^{-1} d^{-1}. The highest average

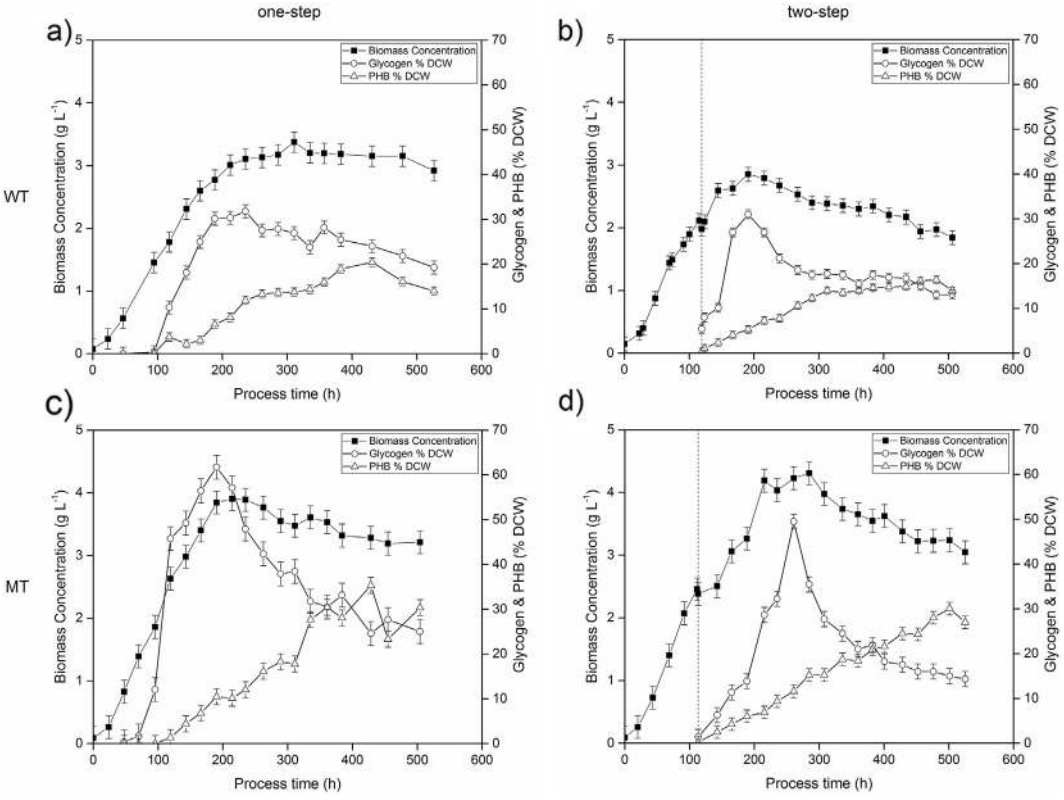


Fig. 3. One-step vs. two-step cultivation of mutant MT_a24 and wild-type PCC 6714. a) & c) represent growth curve, glycogen and PHB content in % DCW for a one-step cultivation of a) wild-type *Synechocystis* sp. PCC 6714 and c) mutant MT_a24. b) & d) demonstrate growth curve, glycogen and PHB content in % DCW for a two-step cultivation of the b) wild-type *Synechocystis* sp. PCC 6714 and d) the mutant MT_a24. The values are derived from biological duplicate cultures. Deviation from the duplicate average is indicated by the error bars.

Table 3
Kinetics and stoichiometric parameters divided into three different phases during PHB production processes of wild-type *Synechocystis* sp. PCC 6714 and mutant MT_a24. The average specific growth rate is given as the average values obtained between two consecutive sampling points during a certain phase of cultivation. The average volumetric production rates are calculated as the average amount of biomass, glycogen and PHB formed over the course of time per liter of culture media in every phase of the cultivation. The average specific production rate is calculated as the average value obtained for PHB or glycogen formed by the biomass in the reactor between two consecutive points over the course of time, calculated for every phase of the cultivation. The average substrate specific uptake rate is calculated as the amount of nitrogen or phosphorus utilized for biomass production over a course of time during a particular phase of the process. Data represent the mean ± standard deviation of three individual measurements.

	one-step cultivation		two-step cultivation		three-step cultivation (2×-Phosphate)		three-step cultivation (3×-Phosphate)	
	WT_PCC 6714	MT_a24	WT_PCC 6714	MT_a24	WT_PCC 6714	MT_a24	WT_PCC 6714	MT_a24
Phase-I								
$\bar{r}_{biomass}$ (mg L^{-1} d^{-1})	190 ± 20	275 ± 30	235 ± 20	410 ± 40	650 ± 50	**660 ± 50**	650 ± 50	600 ± 45
$\bar{\mu}$ (d^{-1})	1.0 ± 0.1	**1.2 ± 0.1**	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
$\bar{r}_{glycogen}$ (mg L^{-1} d^{-1})	NP	**1.0 ± 0.1**	NP	NP	NP	NP	NP	NP
$\bar{q}_{glycogen}$ (mg g^{-1} d^{-1})	NP	**1.2 ± 0.2**	NP	NP	NP	NP	NP	NP
$\bar{r}_{PHB}$ (mg L^{-1} d^{-1})	NP	NP	NP	NP	NP	NP	NP	NP
$\bar{q}_{PHB}$ (mg g^{-1} d^{-1})	NP	NP	NP	NP	NP	NP	NP	NP
$\bar{q}_{phosphorus}$ (mg g^{-1} d^{-1})	**−4.4 ± 0.5**	−3.1 ± 0.5	−2.6 ± 0.5	−3.3 ± 0.5	−3 ± 0.5	−3.9 ± 0.4	−3.8 ± 0.4	−4 ± 0.5
$\bar{q}_{nitrogen}$ (mg g^{-1} d^{-1})	−242 ± 30	−210 ± 20	−260 ± 20	−262 ± 20	−250 ± 20	−260 ± 30	256 ± 30	**−280 ± 30**
Phase-II								
$\bar{r}_{biomass}$ (mg L^{-1} d^{-1})	363 ± 40	**503 ± 50**	148 ± 15	231 ± 20	61 ± 5	44 ± 3	28 ± 3	86 ± 9
$\bar{\mu}$ (d^{-1})	0.18 ± 0.02	**0.26 ± 0.03**	0.05 ± 0.01	0.1 ± 0.01	0	0.03	0	0.004
$\bar{r}_{glycogen}$ (mg L^{-1} d^{-1})	141 ± 15	**395 ± 40**	177 ± 18	299 ± 25	162 ± 15	135 ± 10	139 ± 15	148 ± 15
$\bar{q}_{glycogen}$ (mg g^{-1} d^{-1})	59 ± 6	**140 ± 10**	70 ± 7	78 ± 8	56 ± 6	45 ± 5	47 ± 5	58 ± 6
$\bar{r}_{PHB}$ (mg L^{-1} d^{-1})	61 ± 5	**67 ± 5**	30 ± 5	55 ± 6	48 ± 5	46 ± 5	48 ± 5	53 ± 5
$\bar{q}_{PHB}$ (mg g^{-1} d^{-1})	**23 ± 3**	20 ± 3	12 ± 2	19 ± 2	15 ± 2	15 ± 2	16 ± 2	17 ± 2
$\bar{q}_{phosphorus}$ (mg g^{-1} d^{-1})	ND	ND	ND	ND	1.3 ± 0.2	1.1 ± 0.1	1 ± 0.1	1.5 ± 0.2
$\bar{q}_{nitrogen}$ (mg g^{-1} d^{-1})	ND	ND	ND	ND	ND	ND	ND	ND
PHB (mg L^{-1})	370 ± 30	**403 ± 35**	152 ± 10	383 ± 30	242 ± 20	228 ± 20	234 ± 20	265 ± 25
Phase-III								
$\bar{r}_{biomass}$ (mg L^{-1} d^{-1})	−20 ± 2	−58 ± 6	−78 ± 8	−130 ± 10	−150 ± 20	−128 ± 10	−128 ± 10	−61 ± 5
$\bar{\mu}$ (d^{-1})	0	0	0	0	0	0	0	0
$\bar{r}_{glycogen}$ (mg L^{-1} d^{-1})	−48 ± 4	−143 ± 15	−50 ± 5	−110 ± 10	−39 ± 4	−20 ± 2	−30 ± 3	**5 ± 0.5**
$\bar{q}_{glycogen}$ (mg g^{-1} d^{-1})	−15 ± 2	−39 ± 4	−20 ± 2	−40 ± 5	−17 ± 2	−8 ± 1	−12 ± 2	**0.4 ± 0.05**
$\bar{r}_{PHB}$ (mg L^{-1} d^{-1})	4.3 ± 1	**52 ± 5**	14 ± 2	40 ± 4	8 ± 1	15 ± 2	2 ± 0.5	10 ± 2
$\bar{q}_{PHB}$ (mg g^{-1} d^{-1})	5.4 ± 1	**13.8 ± 2**	2.4 ± 0.5	6 ± 1	1.3 ± 0.5	6 ± 0.5	1 ± 0.1	3 ± 0.4
PHB (mg L^{-1})	273 ± 25	**671 ± 50**	191 ± 10	400 ± 35	305 ± 25	618 ± 50	261 ± 20	401 ± 35
$Y_{PHB/Gly}$ %	28	32	20	22	32	**64**	33	44

Phase-I represents the growth phase of different cultivations when nutrients were abundant.
Phase-II represent the beginning of N and P limitation phase, when glycogen is accumulating as the major storage carbohydrate.
Phase-III or the interconversion phase represent the N and P limitation phase, where glycogen starts to degrade and PHB is produced.
Bold numbers represent the highest value obtained for each parameter for various phases.
NP represents "not produced".
ND represents "not determined".

specific growth rate was determined during the phase-I of the one-step cultivation; for MT_a24 with the value of 1.2 ± 0.1 d^{-1}. The highest average specific phosphorus uptake rate was achieved for the WT_PCC 6714 during a phase-I of a one-step cultivation with the value of 4.4 ± 0.5 mg g^{-1} d^{-1}. However, the highest specific nitrogen uptake rate was observed for the mutant MT_a24 during the growth phase of a three-step cultivation with 3× phosphorus (280 ± 30 mg g^{-1} d^{-1}).

The analysis of phase-II or the glycogen production phase showed that the mutant MT_a24 obtained the highest average volumetric and specific glycogen production rate during a one-step cultivation with the value of 395 ± 40 mg L^{-1} d^{-1} and 140 ± 10 mg g^{-1} d^{-1} respectively. This illustrates that the MT_a24 can achieve more than 200% higher average volumetric and specific glycogen production rate than the wild-type strain. Similarly, the highest average volumetric PHB production rate during phase-II was obtained by MT_a24 with a value of 67 ± 5 mg L^{-1} d^{-1} in a one-step process. The values obtained during phase-II for specific and volumetric PHB production rate were more or less similar for MT_a24 and the parent strain WT_PCC 6714. The analysis of the data obtained for phase-III showed that highest average volumetric and specific PHB production rate was obtained for MT_a24 during a one-step cultivation with values of 52 ± 5 mg L^{-1} d^{-1} and 13.8 ± 2 mg g^{-1} d^{-1} respectively. This values are 3.7-folds and 2.5-fold higher than the maximum values obtained for the WT_PCC 6714. The highest average volumetric PHB production rate for WT_PCC 6714 was obtained during two-step cultivation with a value of 14 ± 2 mg L^{-1} d^{-1} and the highest specific PHB production rate for the WT_PCC 6714 was determined during a one-step process with a value of 5.4 ± 1 mg g^{-1} d^{-1}.

Besides the ability for superior biomass and glycogen production, the MT_a24 shows higher PHB content already during phase-II from CO_2 (previously shown higher CO_2 uptake rates (Kamravamanesh et al., 2018)). However, the major reason for the superiority of the mutant over the wild-type was its greater performance during the phase-III of the PHB production process regardless of the cultivation strategy. Where the MT_a24 obtained a higher PHB yield from the total glycogen produced and therefore a higher PHB productivity.

The analysis of the data derived from three-step cultivations with elevated phosphorus content indicated that the higher phosphorus content improved the performance of the WT_PCC 6714 during phase-I by increasing the average specific growth rate and volumetric biomass production rate. Also during phase-II for glycogen and PHB production rates, similar values for the wild-type and the mutant MT_a24 were obtained. These results may emphasize the importance of phosphorus for cyanobacterial production processes.

In general, the best cultivation strategy with the maximum PHB productivity is the one-step cultivation of mutant MT_a24 when

compared to other cultivation modes.

3.5. One-step process with phosphorus pulses

It has been known that bacterial cells alter their gene expression levels based upon the availability of environmental inorganic phosphate (Vuppada et al., 2018). However, the exact mechanism and role phosphate plays have so far not been discussed. Based on the prior knowledge and the data obtained from the three-step cultivations, it was hypothesized that availability of environmental phosphorus may alter glycogen synthesis in cyanobacteria and maybe the PHB production. Hence, to better understand the role which phosphorus plays during the nutrient starvation phase, one-step cultivations of wild-type *Synechocystis* sp. PCC 6714 and mutant MT_a24 were done manipulating phosphorus concentration in the media using pulses of phosphate, at time intervals when the glycogen content showed a reduction. In order to expose the cells to an early limitation, the one-step cultivation was done with a reduced nitrogen content of 600 mg L^{-1} $NaNO_3$ and 60 mg L^{-1} $K_2HPO_4{\cdot}3H_2O$ as phosphorus supply along with pulses of 0.1 mM phosphorus source at 120 and 210 h of the process. Biomass concentration and the intracellular reserves of carbohydrates were monitored on daily basis.

The results illustrated in Fig. 4a) indicate that cultivation with mutants MT_a24 obtained higher biomass concentration (2.5 ± 0.3 g L^{-1}) when compared to its parent strain wild-type *Synechocystis* sp. PCC 6714 with the maximum biomass concentrations of 2 ± 0.2 g L^{-1}. Addition of phosphate pulses had no significant impact on biomass formation however phosphate pulses increased the glycogen content in both WT_PCC 6714 and the mutant MT_a24 (Fig. 4b). Interestingly, for the wild-type the increase in glycogen content was immediate and steep however for the mutant MT_a24 the recovery occurred gradually. As shown in Fig. 4b) the first phosphate pulse, at 120 h of the process increased the glycogen content in the wild-type from 35% (DCW) to 43% (DCW) after only 6 h and in MT_a24 from 40% (DCW) to 46% (DCW) 50 h after the pulse addition. The second pulse added at 210 h of the process increased glycogen level in the WT_PCC 6714 from 37% (DCW) to 50% (DCW) after 30 h and in MT_a24 from 40% (DCW) to 56% (DCW) after 75 h. The first phosphorus pulse enhanced the specific glycogen production rate for the WT_PCC 6714by 30-folds from 2.4 $mg^{-1}\,g^{-1}\,d^{-1}$ to 76 $mg^{-1}\,g^{-1}\,d^{-1}$ within 6 h. The second phosphate pulse also showed a similar effect on specific glycogen production rate increasing it from −169 $mg^{-1}\,g^{-1}\,d^{-1}$ (negative value represents the glycogen already being degraded) to 130 $mg^{-1}\,g^{-1}\,d^{-1}$ (glycogen production). In the case of the MT_a24 also the same behavior was detected. The specific glycogen production rate was increased from −76 $mg^{-1}\,g^{-1}\,d^{-1}$ to 30 $mg^{-1}\,g^{-1}\,d^{-1}$ after the addition of the first pulse and from −63 to 30 $mg^{-1}\,g^{-1}$ biomass L^{-1} after the second phosphate pulse. In both WT_PCC 6714 and the MT_a24 addition of phosphorus had an immediate impact on specific PHB production rate by reducing it for a short period. It was hypothesized that, as a result of phosphorus addition, the cellular metabolism greatly focused on glycogen synthesis and therefore the PHB production in both strains MT_a24 and WT_PCC 6714 did not reach its maximum expected levels. For MT_a24 only a maximum of 23 ± 2% (DCW) PHB content and for the WT_PCC 6714 a PHB content of 16 ± 2% (DCW) was obtained after 300 h of the process and from that point on both glycogen and PHB showed reduction (Fig. 4c). The results obtained in this work are different from the previously shown data by (Velmurugan and Incharoensakdi, 2018) where they have shown that the glycogen synthesis is independent of PHB synthesis and the overexpression of glycogen biosynthetic genes showed no effect or little effect on the PHB content. This reason for this observation could be presence of other regulatory system in our strain *Synechocystis* sp. PCC 6714.

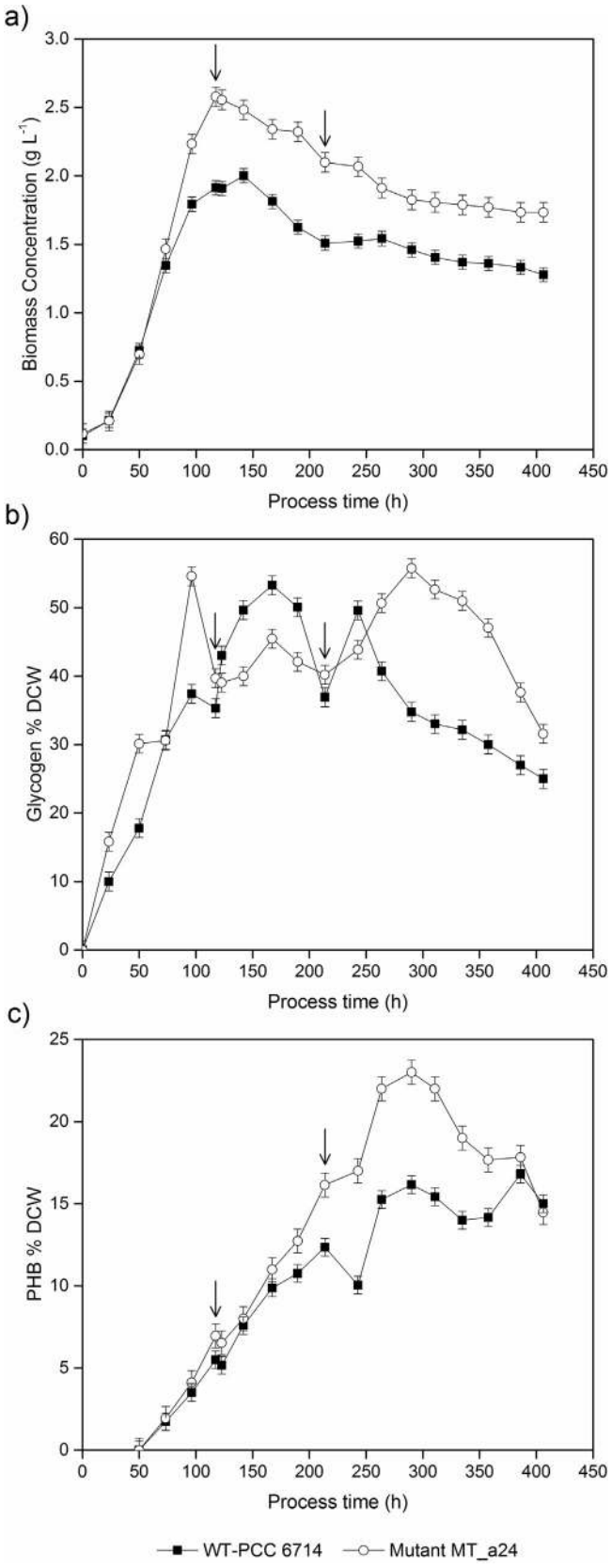


Fig. 4. One-step cultivation with phosphate pulses. a) growth curve b) glycogen content in % (DCW) and c) PHB content in % (DCW) are given for wild-type strain *Synechocystis* sp. PCC 6714 and mutant MT_a24 for a one-step process with phosphorus pulses. Arrows at 120 and 210 h represent the time point where phosphorus (0.1 mM) was added to the process. Error bars represent mean ± SD of triplicate measurements.

3.6. Strategies for boosting the glycogen content

Cyanobacteria accumulate glycogen as a major intracellular carbon and energy storage during photosynthesis (De Porcellinis et al., 2017) when nitrogen is limited. Developments in research have focused on complex mechanisms of glycogen metabolism, concurrently, the target is to redirect carbon from glycogen to desirable products, in this work towards PHB production. The cyanobacterium *Synechocystis* sp. PCC 6714 synthesizes glycogen and PHB simultaneously under nitrogen and phosphorus depleted conditions. Under nitrogen limitation, glycogen is produced at a significantly higher rate than PHB. Later, as limitation persists glycogen synthesis is stopped and glycogen is partially used for PHB production. Here based on the results obtained it was speculated that may be phosphate is required for glycogen synthesis pathway and when the phosphorus reserves are gone the cells are not able to synthesize glycogen anymore and therefore glycogen accumulation is seized. To investigate this hypothesis a one-step cultivation was established using mutant MT_a24 with a limited amount of phosphorus ($1\ g\ L^{-1}$ nitrate and $30\ mg\ L^{-1}$ phosphate). Once the glycogen reached its maximum levels phosphorus was fed to the reactor with the concentration of $0.4\ mg\ g^{-1}$ biomass at the rate of $2.5\ g\ L^{-1}\ h^{-1}$. The phosphorus was given along with 50 times diluted BG-11 media without nitrogen source to avoid dilution of other media sources.

As illustrated in Fig. 5a) glycogen reached a maximum concentration of 49.8% (DCW) at 190 h of the process before the phosphate was fed. Subsequently, the glycogen starts to degrade. Once phosphate was fed the glycogen started to increase and reached a maximum concentration of 76.2% (DCW) at 263 h of the process. After that point, as presented in Fig. 5b) phosphate is not consumed anymore and therefore the residual phosphorous concentration started to increase. The maximum polymer concentration obtained during this time was 25% (DCW) PHB after 310 h of the process. The biomass concentration was more stable during the glycogen synthesis phase and highest biomass concentration obtained was $3.4 \pm 0.4\ g\ L^{-1}$.

The basic principles of cyanobacterial gene expression levels based upon the availability of environmental Pi are already discussed (Westermark and Steuer, 2016). However, the molecular mechanism by which the phosphate is regulating the production of carbohydrates has so far remained unknown. Here in this study, the authors have discovered that phosphorus addition has a direct impact on glycogen biosynthesis in cyanobacterial strain *Synechocystis* sp. PCC 6714, increasing the glycogen levels. The authors hypothesize that phosphorus consumption provides energy for the glycogen metabolism. Wang et al. (2013) have also shown that phosphorus consumption concomitantly increases the intracellular acetyl-CoA pool in engineered 3HB-producing *Synechocystis* sp. PCC 6803. In line with our observations, it could be concluded that under nitrogen and phosphorus limitation the intracellular poly-phosphate is readily used as energy source to produce acetyl-CoA for PHB synthesis and also indirectly for glycogen production. Once poly-phosphate is no longer available the energy required is derived from glycogen conversion to PHB. The conversion of glycogen to PHB is primarily used to gain energy and deliver NADPH (Reis et al., 2003). Therefore in order to maximize glycogen content under nitrogen and phosphorus limiting conditions the best strategy is to wait for utilization of intracellular phosphorus reserves of the cell and then start the phosphate feeding using the average specific phosphorus uptake rate.

In spite of achieving up to $2.6\ g\ L^{-1}$ of glycogen content using phosphate feeding the maximum PHB concentration obtained for MT_a24 was $840\ mg\ L^{-1}$ corresponding to the maximum yield of around 20% from the total intracellular glycogen content. This value is lower than the yield earlier observed for this strain and it can be suspected that the remaining glycogen is utilized so the cell can sustain maintenance. While our understanding of glycogen metabolism has expanded significantly, yet the detailed kinetics of glycogen conversion to PHB in order to gain 100% conversion needs to be investigated.

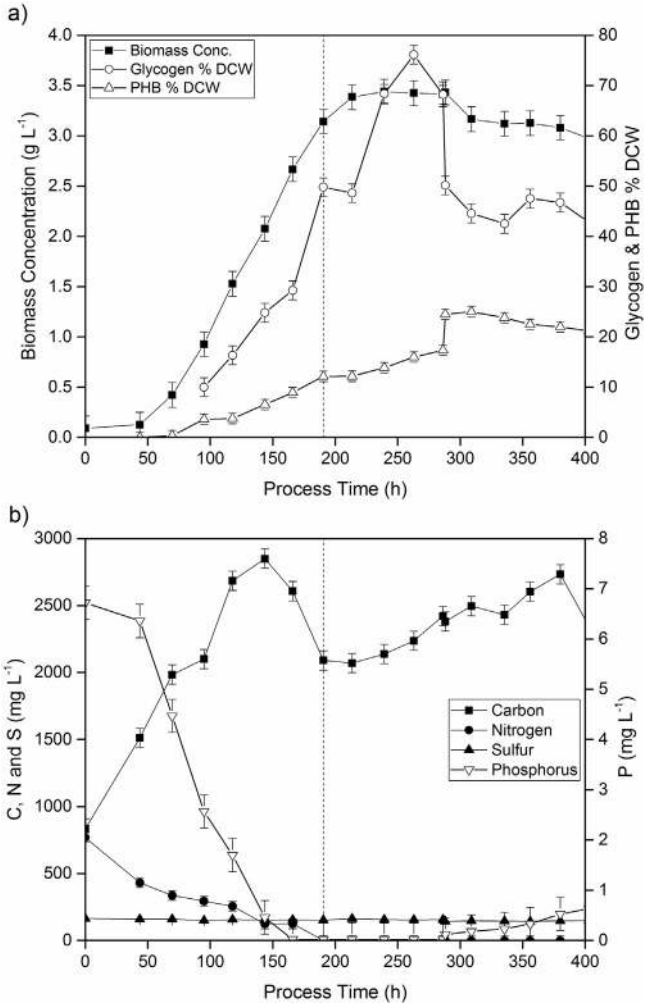


Fig. 5. The fed-batch cultivation of MT_a24. a) The growth curve, glycogen and PHB content in % (DCW) and b) the elemental analysis for the mutant MT_a24 are shown under fed-batch cultivation strategy. The vertical line represents the start of the feeding process. Data represent mean ± SD of duplicate measurements.

Multi-omics analysis can be done to obtain insight into the interaction of PHB and glycogen metabolic pathways. Target genes can be then identified and metabolic engineering can be applied to over-express key enzymes and regulators of PHB and glycogen accumulation and conversion.

Here, the data suggests that as a result of higher intracellular phosphorus concentration the MT_a24 remains superior to the wild-type strain *Synechocystis* sp. PCC 6714 showing excelling performance both during phase-II and phase-III of the PHB production process. During phase-II higher glycogen and PHB synthesis are observed for MT_a24. This act can be justified by the previously shown higher CO_2 uptake rates for MT_a24 (Kamravamanesh et al., 2018). During the phase-III, higher PHB yield from glycogen and yet PHB production from CO_2 is detected for MT_a24. The labeling studies performed by Dutt and Srivastava (2017) has shown carbon fixed following nitrogen deprivation contributes only to 26% of the total carbon for PHB synthesis and the remaining 74–87% of the carbon is obtained from intracellular carbon recycling for PHB synthesis. In this current study, it was observed that for both MT_a24 and wild-type PCC 6714 this ratio depends mostly on the operation mode and PHB formation from CO_2. The PHB yields obtained here from CO_2 are therefore higher than previously shown for cyanobacteria.

In this study, various strategies were systematically applied to

increase production of carbohydrates namely glycogen and PHB in the mutant cyanobacterial strain MT_a24. Our results obtained emphasizes the one-step cultivation with optimized nitrogen and phosphorus content is the best cultivation strategy to maximize the PHB content both for wild-type as well as mutant MT_a24.

4. Conclusions

This study aims at using bioprocess improvement tools to increase sustainable carbohydrate production in cyanobacteria. Using media optimization, a one-step easily scalable bioprocess was optimized for industrial production of PHB. The transient phosphorus availability was demonstrated to improve carbohydrate production efficiency mainly in terms of glycogen production in mutant MT_a24. Using a tuned feeding strategy the maximum PHB and glycogen content acquired for mutant MT_a24 was 1.16 g L^{-1} and 2.6 g L^{-1} respectively. In summary, this work shows the potential of glycogen enriched MT_a24 for bio-refinery and as feedstock for the production of energy and value-added compounds, such as PHB.

Acknowledgments

The authors would like to thank Oliver Spadiut for his valuable input during the experimental design. They also thank Carina Brunnhofer for determination of elemental compositions using ICP-OES.

Author's contributions

DK designed and performed the experiments, analysis, and interpretation of the data, and wrote the manuscript. CS helped with the experimental design, data analysis and structuring of this manuscript. AL supervised the analysis of macronutrients. ML was responsible for the initiation of the study and contributed to the manuscript writing. CH was responsible for the initiation and supervision of this work and supported the experimental planning. All authors read and approved the final manuscript.

Funding

Financial support was gratefully provided by the "Vienna Business Agency", [ID 1890571, 2017].

Availability of data and materials

The data sets supporting the conclusions of this article are included in the main article. The raw data would remain confidential and will not be shared due to a filed patent application (No. A68/2018).

Declaration of conflict of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.11.025.

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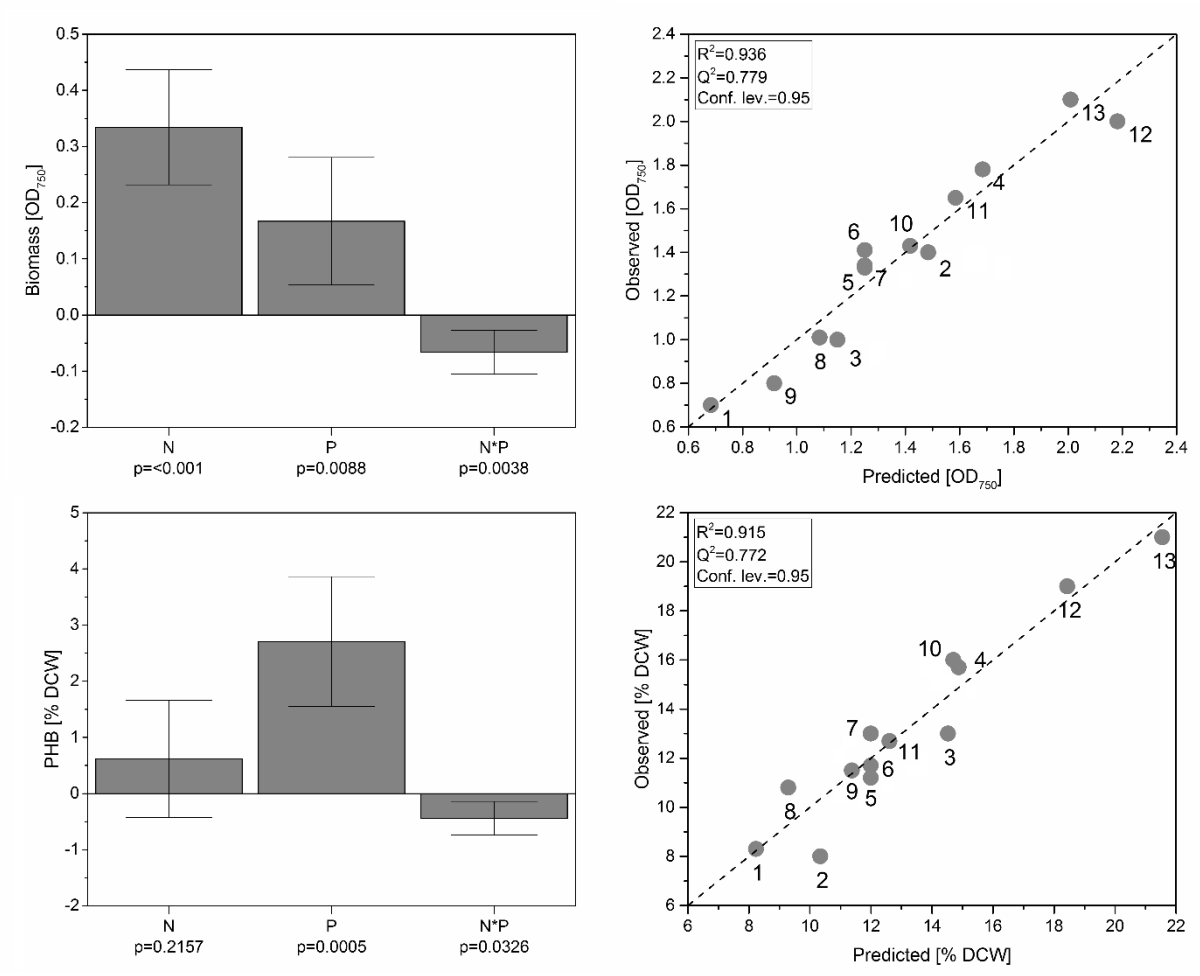
Appendix



Supplementary data- Fig. 1. Coefficient and observed-versus-predicted plots a) & c) represent the coefficient plots for the DoE experiments of *Synechocystis* sp. PCC 6714 a) for biomass concentration and c) PHB accumulation respectively. b) & d) show the model parameters and observed-versus-predicted plots of the models obtained for biomass concentration (b) and PHB accumulation (d) for optimization of nitrogen and phosphorus concentration in a one-step process

5.4 Scale-up challenges and the quality assessment of photosynthetic PHB

5.4.1 Scale-up challenges and requirement of technology-transfer for cyanobacterial poly (3-hydroxybutyrate) production

Abstract: Although extensive research has been directed towards optimization of the strain, media, and the process, yet there is scarce knowledge on the performance and viability of large-scale PHB production lines exploiting cyanobacteria. In this study, the scale-up challenges associated with photosynthetic PHB production are listed. The high PHB producing cyanobacterial mutant, MT_a24, a randomly mutated strain of *Synechocystis* sp. PCC 6714 has been tested in pilot-scale trials for photosynthetic PHB production under outdoor, unsterile conditions. The MT_a24 obtained PHB content of 0.356 g L^{-1} and 1.7 g L^{-1} of glycogen from CO_2 after 10 days of cultivation using a self-limiting media and non-optimized cultivation parameters. The results obtained here indicate that in order to achieve high PHB productivity and to overcome the existing scalability issues reassessment of the optimized parameters in the lab needs to be done during the pilot-scale trials.

This manuscript was submitted as a short-communication to the journal of Bioresource Technology.

Kamravamanesh Donya, Kiesenhofer Daniel, Fluch Silvia, Lackner Maximilian, and Herwig Christoph. Scale-up challenges and requirement of technology-transfer for cyanobacterial poly (3-hydroxybutyrate) production. Journal of Bioresource Technology. Under revision.

Scale-up challenges and requirement of technology-transfer for cyanobacterial poly (3-hydroxybutyrate) production in industrial scale

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Keywords

Cyanobacteria, Poly (3-hydroxybutyrate) (PHB), mutant MT_a24, scale-up, technology-transfer, carbon dioxide (CO_2)

Highlights

- Pilot-scale trials aid in the selection of the significant criteria for scale-up
- Mutant MT_a24 has a potential for PHB production under unsterile outdoor conditions
- Optimization studies performed in lab-scale may not apply to the pilot production
- Cyanobacteria need to be adapted to the outdoor conditions prior to scale-up
- During scale-up media plays an important role in obtaining high productivities

Abstract

Even though extensive research has been directed towards optimization of the strain, media, and the process, yet there is limited knowledge on the performance and viability of large-scale PHB production lines exploiting cyanobacteria. In this study, the scale-up challenges associated with photosynthetic PHB production are listed. The high PHB producing cyanobacterial mutant, MT_a24, a randomly mutated strain of *Synechocystis* sp. PCC 6714, has been tested in pilot-scale trials for photosynthetic PHB production under outdoor, unsterile conditions. The MT_a24 obtained PHB content of 0.356 g L^{-1} and 1.7 g L^{-1} of glycogen from CO_2 after 10 days of cultivation using a self-limiting media and non-optimized cultivation parameters. The results obtained here suggest that in order to achieve high PHB productivity values of the lab and to overcome the existing scalability issues reassessment of the optimized parameters needs to be performed during the pilot-scale trials.

1. Introduction

Microalgae are an alternative sustainable renewable production system, which does not need arable land for cultivation (Jaeger, 2015). Poly (3-hydroxybutyrate) (PHB) is a widespread intracellular storage compound found in prokaryotic organisms (Melnicki et al., 2009), which can be used for the production of biodegradable plastics. Cyanobacteria are well-known for their ability to produce PHB from sustainable resources CO_2 and sunlight (Knoot et al., 2017). The use of PHB derived photosynthetically is not economically feasible due to high production costs. The price of cyanobacterial PHB can be reduced to make the material more competitive in the market either by increasing the productivities or reducing the cultivation costs. This, in turn, can be achieved by smart engineering solutions and physiological adaptations of production strains (Jaeger, 2015).

PHB biosynthesis in cyanobacteria has been enhanced using optimization of cultivation conditions and multi-stage cultivation process which involves nitrogen or phosphorus limitation and the addition of sugars or organic acids, the approaches which did not exploit the photosynthetic potential of cyanobacteria (Wang et al., 2013). As an alternative way to increase productivity, cyanobacteria have been engineered, however, these attempts have shown little success. Recently, the optimization of the acetoacetyl-CoA reductase ribosome binding site in *Synechocystis* led to increase in (R)-3-hydroxybutyrate production of up to 1.84 g L^{-1} in 10 days from CO_2 and the highest productivity of 263 mg L^{-1} d^{-1} was obtained (Wang et al., 2018). As a substitute approach, random mutagenesis has also been used to obtain superior cyanobacterial strains in terms of growth and productivities. The authors previously showed the cyanobacterial strain MT_a24, a UV-mutated strain of *Synechocystis* sp. PCC 6714 produces PHB of up to 37 ± 4 % dry cell weight (DCW) under nitrogen and phosphorus limitation showing the highest productivity of 134 mg L^{-1} d^{-1} (Kamravamanesh et al., 2018). It was also shown that media optimization can be used to increase PHB content in MT_a24 to up to 1.16 g L^{-1}

(Kamravamanesh et al., 2019). Although extensive research has been directed towards optimization studies, yet there is scarce knowledge on the performance and viability of large-scale photosynthetic PHB production lines.

After an initial estimation of the costs to see whether production is economically justifiable, cultivation should be tested in a pilot plant. The pilot-plant gives results concerning "time-space-yield" relationships and economic factors, also aiding in the selection of the criteria for scale-up. So far there exist a few reports on pilot-scale cyanobacterial PHB production under unsterile conditions. Troschl et al. in 2018 reported the production of 125 mg L^{-1} PHB in a 200-liter tubular photobioreactor using *Synechocystis* sp. CCALA192.

For a successfully integrated scale-up, the utmost importance is the proper technology-transfer from the laboratory to industry. The task includes the elucidation of crucial information to be transferred from R & D to pilot-production and for development of the existing process to the production in industrial-scale (Millili, 2011).

The challenges associated with the scale-up of microalgae cultures have already been discussed by various authors (Borowitzka & Avigad, 2017; da Silva & Reis, 2015). However, how to transfer and translate the specifications from lab to industry for cyanobacterial production systems has not been yet described.

In this current study, the authors aim at filling the knowledge gap between the laboratory and the industry showing sustainable and renewable production of PHB from CO_2. This work will provide the scale-up scenario and the preliminary steps towards industrial production of cyanobacterial PHB, in order to facilitate understanding and characterization of the large-scale production and the necessary steps towards commercial production.

2. Material and methods

2.1 Strain and inoculum preparation

The strain MT_a24, a high PHB producing strain of *Synechocystis* sp. PCC 6714 was generated by UV-mutagenesis using the method described by Kamravamanesh et al. in 2018. For inoculum preparation complete BG-11 media was supplemented with 10 mM HEPES buffer, pH 8.2 and 5 mM $NaHCO_3$ was used as a carbon source prior to inoculation. The self-limiting media for PHB production was a modified BG-11 media containing 1000 mg L^{-1} $NaNO_3$ and 60 mg L^{-1} K_2HPO_4 optimized previously and described by Kamravamanesh et al. in 2019.

2.2 Determination of growth, glycogen, and PHB

Biomass growth was determined spectrophotometrically at 750 nm using a UV-Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 6-8 hour intervals.

Glycogen and PHB quantification and determinations were performed using a protocol which is previously described by Kamravamanesh et al. in 2018.

2.3 Bioreactor cultivations

Lab-scale bioreactor experiments were carried out under sterile conditions and light/dark cycles of 16:8 in a 1.5 L jacketed glass reactor with a working volume of 1 L (Applikon B.V, the Netherlands) as previously described by Kamravamanesh et al. (2017). The temperature was maintained at 28 °C and pH was measured with a pH-electrode (Mettler Toledo GmbH, Vienna, Austria) and was automatically maintained at 8.5 by the addition of 0.5 M HCl or 0.5 M NaOH. Agitation speed was at 300 rpm. Gas flow was controlled by mass flow controllers for air and CO_2 (Brooks Instrument, Matfiels, USA). The reactor was bubbled with a mixture of sterile filtered air and 2 % CO_2 at a flow rate of 0.02 vvm (20 mL min^{-1}). The illumination was done using LED strips wrapped around the reactor vessel providing a light intensity of 50 µmol m^{-2} s^{-1} photons in PAR.

The Pilot-scale cultivations were performed outdoors in a 40-liter glass, the tubular hanging garden system at ecoduna AG in Bruck an der Leitha, Austria under non-sterile conditions. The pH was controlled at 8.5 using CO_2 sparging and the circulation was done using a pump.

All fermentation parameters and variable pump set-points in the lab were controlled using the process information management system Lucullus online monitoring system 3.2 (Securecell AG, Schlieren, Switzerland).

Photobioreactor experiments were performed in duplicates and one is shown as an example. Samples were taken in triplicates at 24-hour intervals and were analyzed for biomass concentration, glycogen, and PHB content.

3. Results and discussions

2.1 Technology-transfer for scale-up

The scientific and technical feasibility of photosynthetic PHB production in the laboratory has already been endorsed. The question lies, however, in whether the technology can be supported and further developed to overcome existing scalability challenges to facilitate economic viability (Gendy & El-Temtamy, 2013).

Scaling-up in cyanobacterial processes have commonly been done by a factor of 10, for instance from 100 mL shake flasks to a 1 L reactor and from 1 L to 10 L reactor (Borowitzka & Avigad, 2017). However, the subsequent scale-up can be complex while the quality of the raw material used, production mode, system or critical process parameters also the performing equipment is changed. There are certain guidelines, which need to be considered for a systematic scale-up.

The first step towards scale-up is the estimation of the production scale in order to be commercially and economically relevant. The reactor volume required for the production of 10 tons of PHB per year, assuming the highest reported productivity for the phototrophic cyanobacterial PHB production value of 263 mg L^{-1} d^{-1} (Wang et al., 2018) and annual

operation time of 365 days is around 120 m^3. The assumptions used here are completely optimistic and so far only one report, that also in a lab-scale has achieved such titers (Wang et al., 2018). In case the volumetric productivity is half of the mentioned value the reactor size must be doubled, also for commercial production 10 tones product per annum is used for high-value products and for the commodity, production of more than 1000 tons annually is considered commercial scale (Borowitzka & Avigad, 2017). The assumptions also do not include the efficiency of downstream processing comprising of biomass harvesting, PHB recovery, and product loss during extraction and purification or the risk of contamination or equipment breakdown. Moreover, in many locations, seasonal climate changes do not allow 365 days of production (Borowitzka & Avigad, 2017). Furthermore, the water requirement for such cultivation should be considered taking in to account the water loss from the pond/photobioreactor due to evaporation. Zittelli et al. in 2013 have reported that the cost of building photobioreactor facilities can vary from 10 to 5000 € m^{-2} depending mainly on the size and reactor type. Thus, to prevent future bankruptcy the step by step scale-up planning needs to be performed. In this context, therefore, it is of primary importance to validate the lab-scale quantitative approach first in a pilot-plant with the goal to mimic the final production and operation mode.

2.2 Technology-transfer specifications

Establishment of the most efficient and inexpensive process for the production of PHB in cyanobacteria is only achievable when the issues of mass cultivations which are listed below are acknowledged beforehand.

2.2.1 The cultivation system

The main objective of scale-up is to enlarge the production quantities with similar or higher productivity and product quality (da Silva & Reis, 2015). The most widely used systems for large-scale photosynthetic production are the moderately controlled, sophisticated photobioreactor systems (PBRs) and the industrially applied open-raceway ponds. The closed

PBR systems, have several drawbacks over open raceway systems (oxygen accumulation, cell damage by shear stress, over-heating and the difficulty to scale-up) (Tredici, 2007). As a result of these limitations, the PBRs are expensive to design, build and to operate, and the production cost of the biomass in PBRs may be one order of magnitude higher than in open ponds (Tredici, 2007). On the other hand, the limitation for using genetically modified strains and maintaining monocultures in open systems remains as the main disadvantage of race-way ponds. Irrespective of the cultivation system used, there are certain technical and biological problems which need to be explained for successful scale-up after the detailed quantitative analysis of the process is performed in the lab.

2.2.2 Light

Light availability is one of the most important aspects of the photosynthetic operation. The light intensity and duration directly affect the photosynthetic efficiency of the microalgae biomass and has an influence on the biochemical composition of the cells and productivity (Krzemińska et al., 2014).

In all cultivation facilities, the growth of algae is limited by the shape and the dimensions of the cultivation vessel. Therefore, cultivation facilities have to be designed such that the light conversion efficiency is maximized, which means the use of dense cultures fully absorbing the delivered light (Richmond, 2004). Unfortunately, the steep light gradient formed in these cultures results in overexposure of the upper layers of the culture and leads to low efficiency of light conversion (Nedbal et al., 1996; Richmond, 2004). To prevent prolonged light saturation, cultures have to be rapidly mixed (Nedbal et al., 1996). The photorespiration is a competing process to carboxylation, where the organic carbon is converted into CO_2 without any metabolic gain (Richmond, 2004). Under high or low irradiance, a high concentration of oxygen and reduced CO_2 levels, the reaction equilibrium is shifted towards the photorespiration, as a result reducing the productivity (Richmond, 2004). Therefore, in order to obtain optimum yield in microalgae mass cultivations, it is of utmost importance to minimize the effect of

photorespiration. Furthermore, the influence of the day/night regime on the process and the productivity vary significantly from the dark/light cycles mimicked in the lab. In **Fig. 1** the growth of cyanobacterial mutant strain, MT_a24 (Kamravamanesh et al., 2019) on a self-limiting media in lab-scale and pilot-scale reactors is represented by optical density (OD) measurements at 750nm. Both cultivations show a very similar behavior until 180 hours of the process after that the flattening of growth for the process in lab-scale is observed. The stationary phase represented for the lab-scale process means the growth is seized as a result of nutrient starvation indicating the polymer synthesis phase. Although the cultivation conditions of outdoor culture are neither optimal nor controlled, the mutant MT_a24 obtains slightly higher OD_{750} for the outdoor cultures. However, fluctuation in OD_{750} is observed between the measurements performed in the morning and in the evening in outdoor cultures, which could be due to the changes in the irradiance spectrum of day/night regime. It has been known that even dense cultures of microalgae are exposed to large fluctuations in light intensity due to the daylight variations and the poor mixing (Richmond, 2004). Therefore, it is of maximum importance to previously expose and adapt the cells to the day/night regime instead of routinely done dark/light cycles before starting the pilot-scale trials.

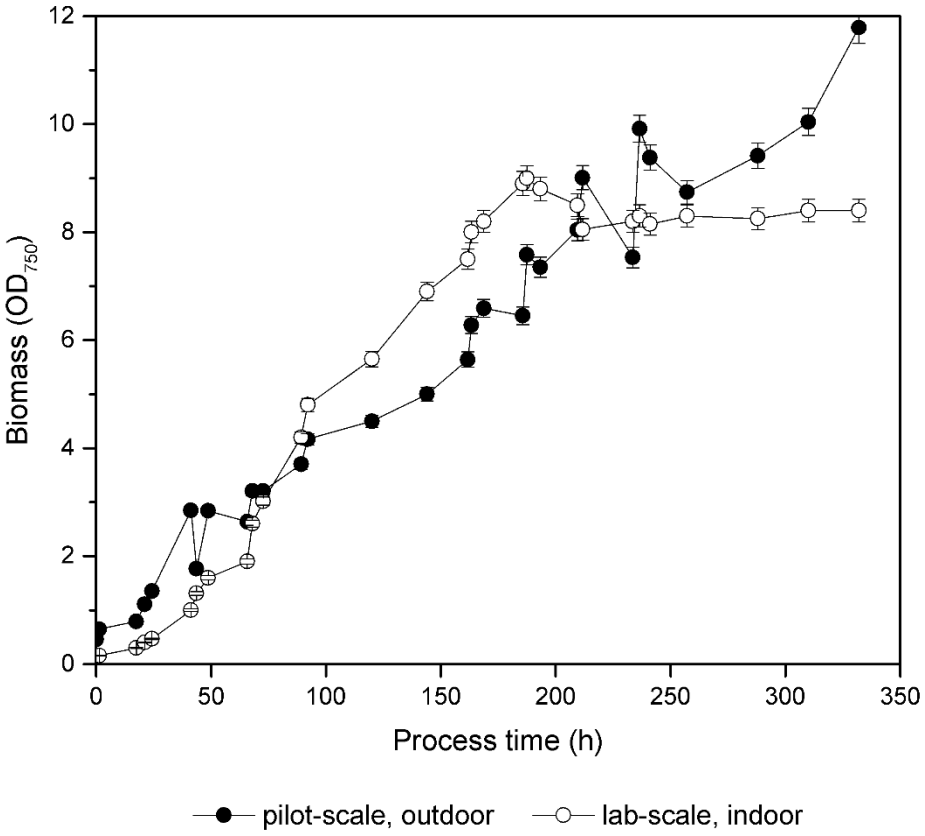

Fig. 1. Represents the phototrophic growth of cyanobacterial strain MT_a24 on a self-limiting media under controlled defined conditions in an indoor one-liter photobioreactor and under unsterile outdoor conditions of a 40-liter pilot-scale tubular photobioreactor. Data represent the mean ± SD from three independent measurements.

2.2.3 Temperature control

Maximum productivity can be obtained under optimal cultivation conditions. The authors previously showed the significance of temperature on growth and PHB productivity in the parent strain *Synechocystis* sp. PCC 6714 (Kamravamanesh et al., 2017).

Commonly, the outdoor cultures of microalgae are exposed to a range of environmental stresses. The most frequent combination is light and temperature fluctuations (Richmond, 2004). The fluctuations in light intensity occur mainly in a range of 1-2 hours, the increase in temperature, however, is a slower process and occurs in a much longer period. This kind of de-synchronization, which affect photosynthesis and growth of outdoor algal cultures, results in a

unique stress condition under which photoinhibition can be induced due to the sub-optimal temperature conditions at a relatively low light intensity (Richmond, 2004; Vonshak et al., 2001). While open ponds are limited by low temperatures in the morning, PBRs generally require cooling at midday (Tredici, 2007). Shading, immersion in a water bath and water spraying are the most common solutions adopted to avoid overheating of outdoor PBRs (Tredici, 2007). For that reason, preferably the microalgae production farm needs to be located in places with a moderate climate and minor temperature fluctuations during the night. Moreover, the cultures which are selected for outdoor cultivations need to be versatile or at least adapted to a wide temperature range.

2.2.4 Nutrient limitation

Environmental factors, particularly light, temperature, nutrient status, and salinity, not only affect photosynthetic efficiency and the productivity of microalgae but also influence the pattern, pathway, and activity of cellular metabolism and thus the dynamic cell composition (Qiang, 2004).

Carbon, nitrogen, and phosphorus are the three most important nutrients for autotrophic growth and their supply is key to microalgae biotechnology (Richmond, 2004). Nitrogen and phosphorus are generally the essential constituents of cyanobacteria and play important role in cellular metabolic processes. Under nitrogen and phosphorus limitation, the photosynthetic efficiency falls and the focus is directed towards the accumulation of carbohydrates and fatty acids and in case of some cyanobacteria accumulation of PHB. Nitrate and ammonia are the most widely used nitrogen source in cyanobacterial cultivations. Use of ammonia makes the process control more challenging because the release of H+ ions leads to drastic pH changes, however, the advantage is the lower cost (Qiang, 2004). The authors have previously shown that the nitrogen source used for growth can influence PHB productivity, in the case of *Synechocystis* sp. PCC 6714 (Kamravamanesh et al., 2017) the nitrate use was favorable to the final PHB content.

Fig 2 represents the glycogen and PHB accumulation for strain MT_a24 using a self-limiting media (Kamravamanesh et al., 2019) both in one-liter indoor and 40-liter outdoor pilot-scale reactor. The glycogen content in the outdoor culture (**Fig. 2 a**) showed significant oscillations until the end of the process and reached a value of about 55 % (DCW) corresponding to 1.7 g L^{-1} after 330 hours of the process. As expected, the glycogen accumulation for the indoor one-liter reactor showed a constant increase and attained a maximum value of 49.5 % (DCW) corresponding to 1.4 g L^{-1} after 260 hours of the process and later started to reduce significantly. Also, the highest PHB content (**Fig. 2 b**) in the outdoor culture observed was a value of 11.3 % (DCW) corresponding to 0.365 g L^{-1} in contrast to a maximum of 27 % (DCW) PHB (782 mg L^{-1}) which was obtained for the lab-scale indoor process. While the authors have previously shown the significance of the dark/light cycle on the photosynthetic PHB productivity for the *Synechocystis* sp. PCC 6714 (Kamravamanesh et al., 2017) the PHB content for MT_a24 observed here in the lab-scale under dark/light cycles is lower than previously observed for this strain.

This diversity in the obtained results from outdoor and indoor cultivations could not be easily explained, however, one hypothesis could be the transition between dark/light cycles in the lab leads to a different carbohydrate metabolism than the naturally occurring day/night regime. The other difficulty could be the uncontrolled temperature of the pilot-reactor and the difference in the pH-control system. While PHB being the intracellular carbon reserve material can remobilize and be utilized as an energy source when there is no extracellular carbon source available. However, the main inspection could be the fact that the media has been optimized for the process parameters used in the lab and not the pilot.

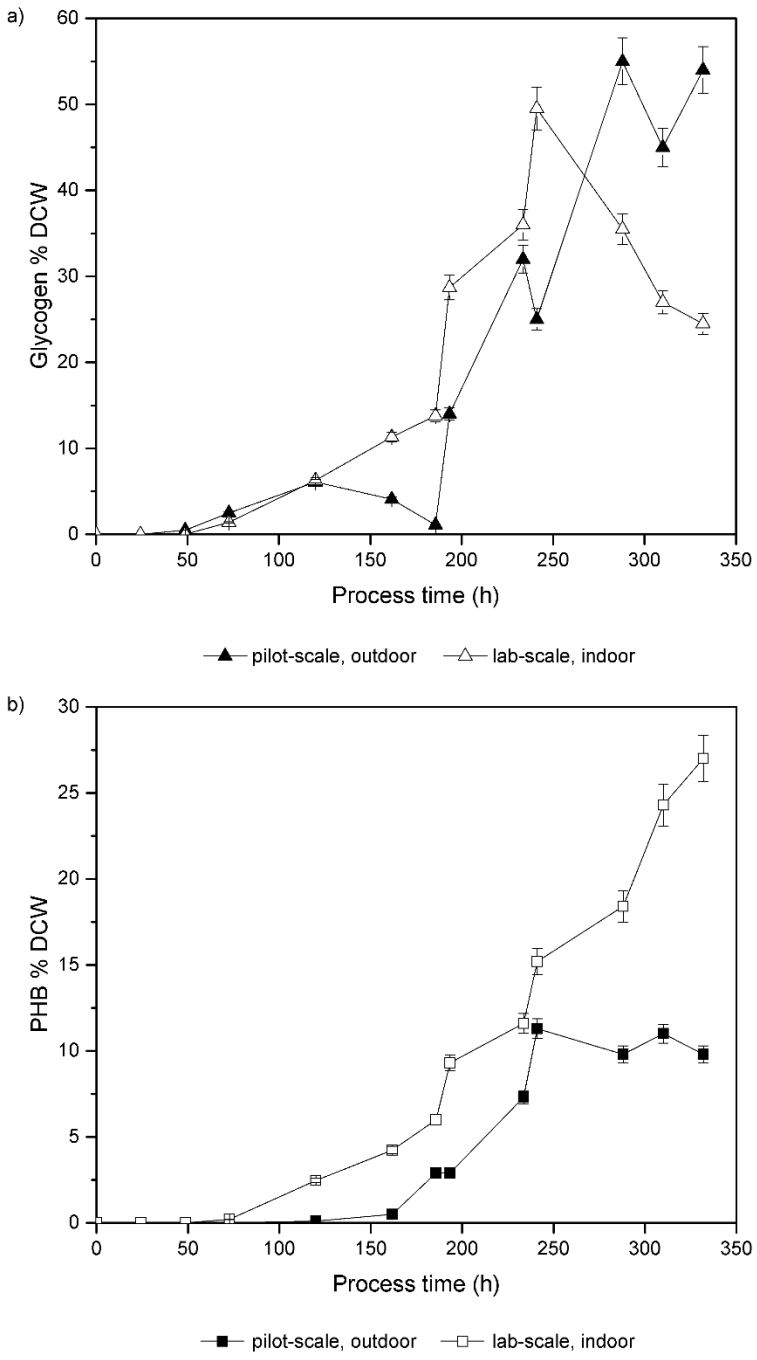

Fig. 2. Photosynthetic cultivation of MT_a24 using a self-limiting media both in a one-liter lab-scale reactor and a 40-liter outdoor pilot reactor a) glycogen accumulation and b) PHB content. Data represent the mean ± SD from three independent measurements.

This phenomenon indicates the complexity of the scale-up operation and hence presents the need for reassessment of all achievable cultivation parameters once moving from the lab to pilot studies. One of the most critical parameters being the media and the quality of water used for

large-scale production. Further, the cost estimation for the media used for microalgae biomass production in a 100 tones process is about 3000 $ tonnes^{-1} (da Silva & Reis, 2015). This implies that making an economically relevant process for the production of cyanobacterial commodity products such as PHB is only possible when alternative cheap sources such as agro-industrial wastewaters with high nitrogen and phosphorus content are applied. It has been shown that a 70-110 tones/(ha annum) microalgae facility using wastewater can result in a saving of 48,400-74,800 $/(ha annum) for nitrogen removal and 4,575-7,625 $/(ha annum) for phosphorus removal (da Silva & Reis, 2015). Media plays as an important factor in cyanobacterial production systems and the productivities which are reported in the lab using the sterile and defined media may significantly differ from the values obtained using alternative resources.

2.2.5 Environmental stress

Controlled PBR systems can prevent fluctuations and variations in the culture. Nevertheless, the difficulty to operate and scale-up and the high production cost of the PBR systems is the main limitation associated with their commercial employment. On the other hand, the use of open raceway ponds due to changes in environmental conditions mainly pH and temperature shifts may result in sudden culture failure.

The pH-static control in large-scale processes is mainly done via the CO_2 sparging, during the photosynthesis the OH^- ions accumulate in the system increase the pH leading to overshoot in CO_2 concentration in the culture and during the respiration, the pH drops leading to little or no CO_2 in the system. Some microalgae become susceptible to invasion by other competing species when growing under slight pH stress (Goldman et al., 1982). It is noteworthy that the sub-optimal environmental conditions may not only reduce the overall biomass or PHB productivity in cyanobacteria but also the potential instability which may lead to contamination and culture collapse. Essentially, the production of PHB in most cyanobacteria occur under nutrient limitation leading to culture instability and therefore the risk of contamination is increased

during the PHB production phase. The sterilization of large surface PBRs is a difficult and expensive task and cultivation under unsterile conditions can be challenging to most lab strains. For some extremophile species, the pH control, extreme salinity or high temperature can be used to maintain the cells during mass cultivations.

Large-scale cyanobacterial processes will surely contribute to the development of a sustainable industry for biomass and PHB production. Many wild-types and genetically modified species of cyanobacteria show potential for large-scale production, however, there is a lack of information on commercial trials. In laboratory-scale, the cyanobacterial performance is mostly evaluated in terms of productivity, whereas issues such as contamination, material degradation, and the sustainability and the reliability of the production process, which more than the yields can determine the success or failure of the large-scale activity, are neglected (da Silva & Reis, 2015). Also, when the specific rates or the yields are compared, the expectations are based on the promising results obtained in the laboratory under sterile conditions which are never attained in large-scale.

Even though the importance of full optimization study in lab-scale before considering the scale-up has been emphasized (Rawat et al., 2013) it seems crucial to reevaluate the optimized parameters during the pilot-scale studies. Provided that the design or shape of the pilot reactor varies from the lab-scale reactor or the water quality, the temperature or pH control differ, the assessment of the parameters and the determination of productivities need to be redone.

More importantly, the research needs to focus in the future to advance the understanding and link between glycogen and PHB synthesis and the carbon partitioning and the switch between glycogen and PHB metabolism. The control of glycogen production and the shift towards PHB synthesis, which finally leads to high titers of the polymer are certainly required. The mutant strain MT_a24 accumulates glycogen of more than 50 % (DCW) (Kamravamanesh et al., 2019) generally from the onset of the nitrogen limitation. PHB is considered as a secondary energy

and electron sink (Stal, 1992) which accumulates mainly at much lower contents. Blocking the glycogen synthetic pathways might not be a practical option as it has already been reported that the glycogen deficient cyanobacteria are impaired with growth and survival (Gründel et al., 2012). In order to achieve high PHB titers during large-scale cyanobacterial cultivations, the pathway needs to be functionally bridged leading to glycogen conversion to produce higher PHB contents. This might be achievable using genetic engineering tools or the modification of process parameters.

The current study tries to fill the gap between industry and research, the main issues regarding tech-transfer for the scale-up scenario of the cyanobacterial PHB production process are discussed here. The possible routes to overcome the bottlenecks of scale-up operation are proposed which may result in sensible preparation for a viable process. The strategies listed are: i) Cyanobacterial cultures need to be adapted to outdoor cultivation conditions (light, temperature, and pH changes) before the start of scale-up process ii) The expected outdoor environment need to be estimated in advance iii) The experiments are required in pilot-scale to mimic the physiological response of the cells to the final large-scale outdoor process iv) The experiments need to be done and productivities have to be determined using the real media and water (waste or recycled water) used for the actual process. Like algae-based industries, PHB production from cyanobacteria requires the optimum combination of technical innovations in systems and processes, coupled with economic feasibility in the practical application and integrated scale-up for industrial production and marketing (Gendy & El-Temtamy, 2013).

4. Conclusions

The cyanobacterial derived bioplastics can be economically feasible and competitive in the market with other bioplastics only with a long term considerable research and development work with the starting point being the pilot-scale production. The optimized strains, synthetic

media and cultivation parameters used under sterile conditions of the lab may not be applicable for large-scale outdoor production systems. The research should focus on the development of more versatile and industrially suitable cyanobacterial production systems considering the challenges of outdoor processes. Further, to obtain the productivity values from the lab in large-scale the re-optimization of media and process seem obligatory.

Declaration of the author's contributions

DKA and CH have planned this study. DKA has carried out the experiments in the lab-scale and DKI performed the experiments in pilot trials with the supervision of SF. DKA has performed the analytics and written this manuscript. The study has been initiated by ML and CH and CH has supervised this project.

Conflict of interest

The authors have no conflict of interest to disclose.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable

Funding

"This research was funded by the authors' institutions".

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5.4.2 Quality assessment of photosynthetically synthesized poly (3-hydroxybutyrate) using a randomly mutated strain of *Synechocystis* sp. PCC 6714

Abstract: Production of value-added compounds from CO_2 feedstocks could help to lower the negative impacts of this greenhouse gas on the environment. Poly (3-hydroxybutyrate) (PHB) is a biodegradable and biobased polymer which can be synthesized sustainably using CO_2. PHB with a high crystallinity represents properties similar to synthetic polyesters and also to polyolefins such as polypropylene. However, the low elongation and break and the brittleness of PHB are limitations which can be overcome by using other PHA, blends of copolymers. Here in this study the PHB produced by cyanobacteria was extracted and characterized using various techniques. The Fourier Transform Infrared spectroscopy (FTIR) showed the characteristic signals expected for the phototrophic PHB. Differential Scanning Calorimetry (DSC) used to determine the thermal characterization of PHB showed a melting temperature of 151,42 °C and 73,11 °C for the crystallization process. From the Gel Permeation Chromatography (GPC) the molecular weight of cyanobacterial PHB was determined to be 1051900 g mol^{-1}.

The data from this work has not yet been published!

Donya Kamravamanesh, Krampl Margit, Itzinger Regina, Paulik Christian, Chodak Ivan, Lackner Maximilian and Herwig Christoph. Quality assessment of photosynthetically synthesized poly (3-hydroxybutyrate) using a randomly mutated strain of *Synechocystis* sp. PCC 6714. Unpublished data

Unpublished data

Quality assessment of photosynthetically synthesized poly(3-hydroxybutyrate) using a randomly mutated strain of *Synechocystis* sp. PCC 6714

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Unpublished data

Abstract

Production of value-added compounds from CO_2 feedstocks could help to lower the negative impacts of this greenhouse gas on the environment. Poly(3-hydroxybutyrate) (PHB) is a biodegradable and biobased polymer which can be synthesized sustainably using CO_2. PHB with a high crystallinity represents properties similar to synthetic polyesters and also to polyolefins such as polypropylene.However, the low elongation and break and the brittleness of PHB are limitations which can be overcome by using other PHA, blends of copolymers. Here in this study the PHB produced by cyanobacteria was extracted and characterized using varioustechniques. The Fourier Transform Infrared spectroscopy (FTIR) showed the characteristic signals expected for the phototrophic PHB. Differential Scanning Calorimetry (DSC) used to determine the thermal characterization of PHB showed a melting temperature of 151.42 °C and 73.11 °C for the crystallization process. From the Gel Permeation Chromatography (GPC) the molecular weight of cyanobacterial PHB was determined to be 1051900 g mol^{-1}.

Keywords

Poly (3-hydroxybutyrate) (PHB), MT_a24, quality assessment, Cyanobacteria, carbon dioxide (CO_2)

Unpublished data

1. Introduction

World's dependency on limited fossil fuels for the production of petroleum-based products such as plastics, makes the commercialization of renewableand sustainable polymers extremely demanding. Polyhydroxyalkanoates (PHAs) are a versatile group of biodegradable polyesters produced by microorganismsaccumulated intracellularly to levels as high as 90 % dry cell weight (DCW) under stressed growth conditions acting as carbon and energy storage material(Madison & Huisman, 1999; Reddy et al., 2003).

The PHA is non-toxic, biocompatible, biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, are highly crystalline, optically active and isotactic (stereochemical regularity in repeating units), piezoelectric and insoluble in water(Reddy et al., 2003). These features make them highly competitive with polypropylene, the petrochemical-derived plastic.

More than 100 different monomer units have been identified as constituents of the storage PHA which creates a possibility for producing different types of biodegradable polymers with an extensive range of properties.(Reddy et al., 2003). In general, PHAs are classified into two groups according to the carbon atoms that comprise their monomeric unit. Short-chain-length PHAs (scl-PHAs) consist of 3–5 carbon atoms, whereas medium-chain-length PHAs (mcl-PHAs) consisting of 6–14 carbon atoms(Kourmentza et al., 2017). Poly(3-hydroxybutyrate) (PHB), the most well-known scl-PHA member, due to biocompatibility and biodegradability, possesses extensive interesting functions and can replace fossil-based plastics in many applications (Ten et al., 2015). However, the low elongation and break and the brittleness of PHB are limitations that can be overcome using other PHA, like blends of copolymers such as polyhydroxyvalerate (PHV) and poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV). The copolymer can either be directly biosynthesized under varying cultivation conditions or be chemically produced in vitro. Apart from short-chain length PHA, there are

medium- and long-chain-length polymers which can help to tailor the material properties (Lackner et al., 2018).

Current industrial PHA production processes rely mostly on the availability of agricultural resources, which are unsustainable (compare the food vs. fuel discussion with first-generation biofuels) and leaving sometimes an ecological footprint (Behler et al., 2018).PHB is commercially produced by heterotrophic bacteria such as *Cupriavidusnecator* (Madison &Huisman, 1999), and recombinant *Escherichia coli*(Schubert et al., 1988). Despite relatively high yields of PHB, production from bacterial fermentation requires sugar supplementation and continuous oxygen supply which results in high substrate and operation costs (Steinbüchel,2008; Wu et al., 2001). PHAs can be produced through sustainable bioprocess engineering and have displayed remarkable flexibility in their physical and chemical properties(Koller, 2016).

As an alternative method, PHB has been produced in cyanobacteria using sustainable resources CO_2 and sunlight. The use of PHB derived photosynthetically is not economically feasible due to the low productivity of the polymer. The price of cyanobacterial PHB can be reduced to make the material more competitive in the market either by increasing the productivities or reducing the cultivation costs.

In this study the polymer produced from the high PHB producing mutant of cyanobacterial strain *Synechocystis* sp. PCC 6714, MT_a24 was extracted using liquid-liquid extraction from the whole cells. The characterization of the polymer was done using various methods such as Fourier Transform Infrared spectroscopy (FTIR) for the chemical characterization whereas Differential Scanning Calorimetry (DSC) was used to determine the thermal characterization and Gel Permeation Chromatography (GPC) was done for the analysis of thermal properties of the polymer.

2. Material and Methods

2.1 Strain and inoculum preparation

An axenic culture of wild-type strain *Synechocystis*sp.PCC 6714 was purchased from Pasteur Culture Collection of Cyanobacteria (Pasteur Institute, Paris, France). Unless stated otherwise, *Synechocystis*sp.PCC 6714 was grown in BG-11 medium (Rippka et al., 1979) supplemented with 10 mM HEPES buffer pH 8.2 with the addition of 15 g L^{-1} of Kobe agar for plates and 5 mM $NaHCO_3$ as carbon source prior to inoculation. In order to induce nitrogen deficiency, cells were cultured in BG-11 media without nitrate and ammonia. Ferrous ammonium citrate and Co $(NO_3)_2.6H_2O$ were substituted with equimolar concentrations of Ferric citrate and $CoCl_2.6H_2O$ in terms of iron and copper content. For phosphorus limitation, KH_2PO_4 was replaced with an equimolar concentration of KCl in terms of potassium content

2.3 Bioreactor cultivations

Lab-scale bioreactor experiments were carried out under sterile conditions and light/dark cycles of 16:8 in a 1.5 L jacketed glass reactor with a working volume of 1 L (Applikon B.V, the Netherlands) as previously described by Kamravamanesh et al. (2017). The temperature was maintained at 28 °C and pH was measured with a pH-electrode (Mettler Toledo GmbH, Vienna, Austria) and was automatically maintained at 8.5 by the addition of 0.5 M HCl or 0.5 M NaOH. Agitation speed was at 300 rpm. Gas flow was controlled by mass flow controllers for air and CO_2 (Brooks Instrument, Matfiels, USA). The reactor was bubbled with a mixture of sterile filtered air and 2 % CO_2 at a flow rate of 0.02 vvm (20 mL min^{-1}). The illumination was done using LED strips wrapped around the reactor vessel providing a light intensity of 50 µmol m^{-2} s^{-1} photonsin PAR.

The Pilot-scale cultivations were performed outdoors in a 40-liter glass, the tubular hanging garden system at ecoduna AG in Bruckan derLeitha, Austria under non-sterile conditions. The

pH was controlled at 8.5 using CO_2sparging also the circulation was done using sterile filtered air.

All fermentation parameters and variable pump set-points were controlled using the process information management system Lucullus online monitoring system 3.2 (Securecell AG, Schlieren, Switzerland).

Photobioreactor experiments were performed in duplicates and one is shown as an example. Samples were taken in triplicates at24-hour intervals and were analyzed for biomass, PHB content.

2.4Determination of the PHB content

PHB quantification was done using the procedure described by Schlebusch and Forchhammer (2010). Pre-weighed dried cells (2-5 mg) were boiled with 1 mL conc. H_2SO_4 at 100 °C on a heating block (Accublock^{TM}, Labnet, USA) for one hour to convert PHB to crotonic acid. Samples were allowed to cool down and subsequently diluted 20 times using 0.014 M H_2SO_4. Crotonic acid was determined using a high-performance liquid chromatography system (Thermo-Fischer Scientific, USA) with a Nucleosil C8 column (Macherey-Nagel, Germany) using an isocratic method. The mobile phase used was 20 mM NaH_2PO_4 buffer; pH 2.5 and acetonitrile (70:30 v/v) with a flow rate of 0.85 mL min^{-1} and a column temperature of 30 °C. Detection of crotonic acid was done using a diode array detector (DAD) detector (Thermo-Fischer Scientific, USA) at 210 nm. For calibration, pure PHB (Sigma- Aldrich, USA) was treated accordingly and analyzed in parallel with samples. Instrument control and peak evaluation were done with Chromeleon 7.2 (Thermo-Fischer Scientific, USA). The percentage PHB per (DCW) was determined by the amount of PHB obtained from HPLC analysis and the cell dry weight of biomass used for the analysis using equation [3]:

$$\%\ \text{DCW}\ PHB = \frac{\text{mg (PHB)}}{mg\ (DCW)} * 100. \quad [3]$$

2.5PHB extraction

The PHB was extracted using a method previously described by Hahn et al. in (1995)with some modifications. The biomass was lyophilized and suspended in chloroform 30 ml g^{-1} of biomass. The suspension was put on a heating block and allowed to boil for one hour on continuous shaking at 300 rpm. The hot suspension was filtered through a Whatmanfilter paper (Merck, Austria). The PHB was extracted using 10 times volume of ice-cold methanol. The polymer was separated using centrifugation at 30,000 rpm and the pellet was air-dried. The PHB was finally washed using cold Acetone.

2.6 Dynamic Scanning Calorimetry (DSC)

The DSC was done using the extracted PHB with a TA instrument DSC Q20 in a heat-cool-heat cycle using the following parameters:

1) Equilibration at -40 °C
2) Isothermal for 5 minutes
3) The ramp from 10 °C min^{-1} to 200 °C
4) Isothermal for 3 minutes
5) Ramp 10 °C min^{-1} to -40 °C
6) Isothermal for 5 minutes
7) The ramp from 10 °C min^{-1} to 200 °C

And for the analysis, the second cycle was used.

2.7 Infrared Spectroscopy

The Attenuated Total Reflection Infrared Spectroscopy was done using a Nicolet 5700 FTIR spectrometer.

2.8 Gel Permeation Chromatography (GPC)

The GPC was performed using an Agilent 1100 chromatography system equipped with a refractive index (RI) response detector. The polymer was dissolved in Chloroform with the

concentration of 2 mg mL^{-1} overnight. The analysis was carried out at 40 °C in chloroform, at the flowrate of 1 ml min^{-1} and injection volume of 100 µL. The GPC system was calibrated prior to analysis using narrow polystyrene standards ranging from 1470 g mol^{-1} to 6035000g mol^{-1}. The weight average molar mass Mw, number average molar mass Mn, and molar mass dispersity D for the measurements were determined from the peaks corresponding to the polymer fraction and are therefore determined as "polystyrene-relative" molecular weights.

3. Results and Discussions

3.1 PHB extraction

Once PHB was extracted from the cells the purity was determined using HPLC. The analysis of extracted PHB from cyanobacteria using chloroform: methanol extraction showed a purity of more than 95 % (DCW).

3.2Dynamic Scanning Calorimetry

The melting temperature and enthalpies of fusion of the PHB extracted from cyanobacteria was determined using DSC. The provided Thermogram in **Fig. 1**shows the distinct characteristic signals for melting, crystallization and the glass transition. The onset temperature accounts to 151.42 °C for the melting temperature (T_m) of the phototrophically synthesized PHB and 73.11 °C for the crystallization process (T_c). The glass-transition temperature (T_g) can be seen in the enlargement of the Thermogram. The temperature at the inflection point accounts to 48.51 °C. The values obtained here suggest the high crystallinity of the extracted polymer.

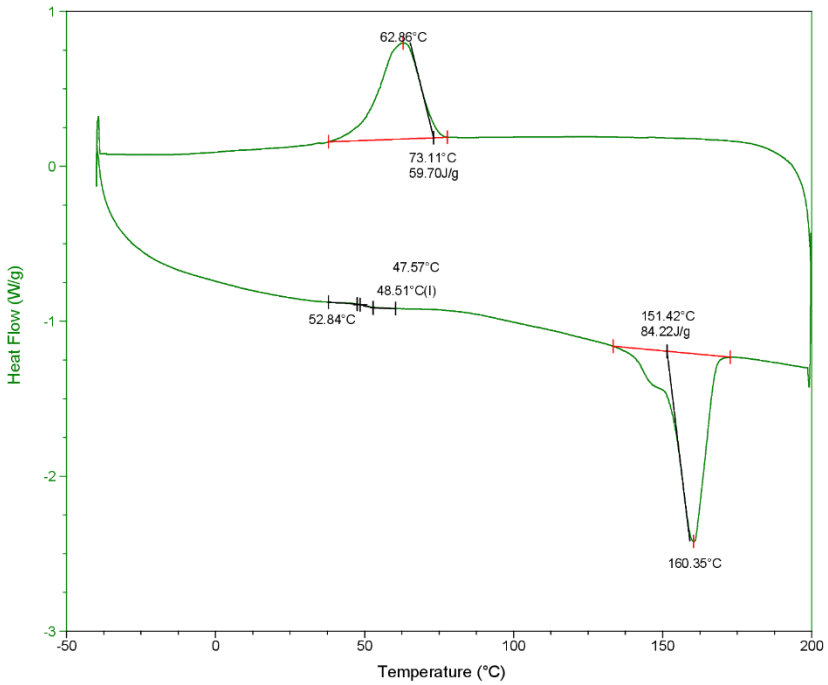
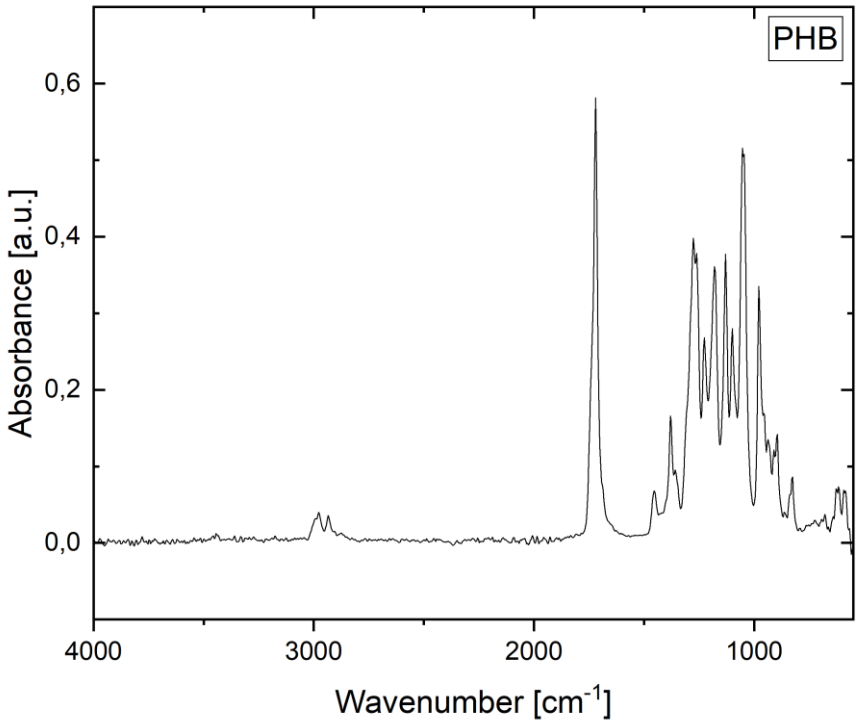


Fig 1. Represents the Thermogram for the DSC determination of photosynthetically produced PHB samples extracted from cyanobacteria.

3.3 Infrared Spectroscopy

The FTIR spectrum for the photosynthetically produced PHB is presented in **Fig. 2 a)**. The spectrum visibly shows the expected characteristic bands for PHB. In order to validate this observation the obtained spectrum is aligned with the PHB spectrum from databases. The stacked signal illustrated in **Fig. 2b)**suggests the similarity of the measured PHB sample from cyanobacteria and the specimen from the database.

a)



b)

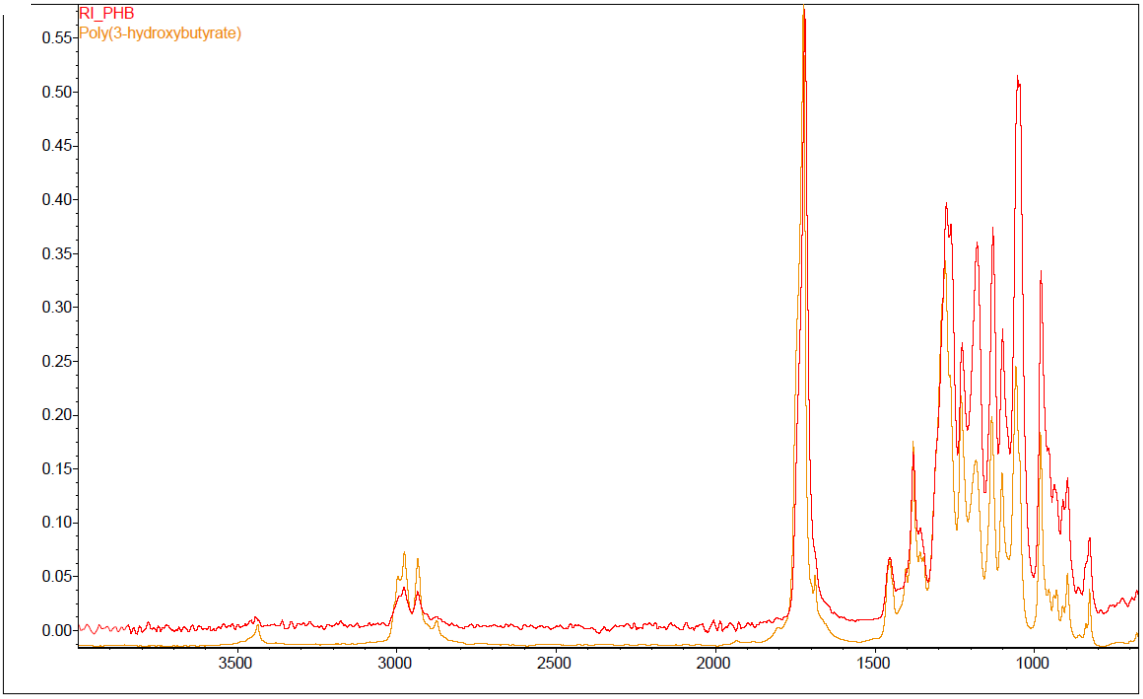


Fig 2. Represents the spectra for the FTIR determination of a) PHB extracted from cyanobacteria and b) The stacked spectra of determined cyanobacterial PHB shown in red and the spectra of PHB provided by HR Hummel Polymer and Additives Database in yellow.

3.4 Gel Permeation Chromatography (GPC)

The GPC analysis (**Fig. 3**) of PHB produced from CO_2 using cyanobacteria showed the molecular weight was (Mw = 1051900 g mol-1) and the number average of the molecular weight of the PHB was (Mn = 316060 g mol^{-1}). The polydispersity index (Mw/Mn) of the cyanobacterial PHB was determined to be (D = 3.328).

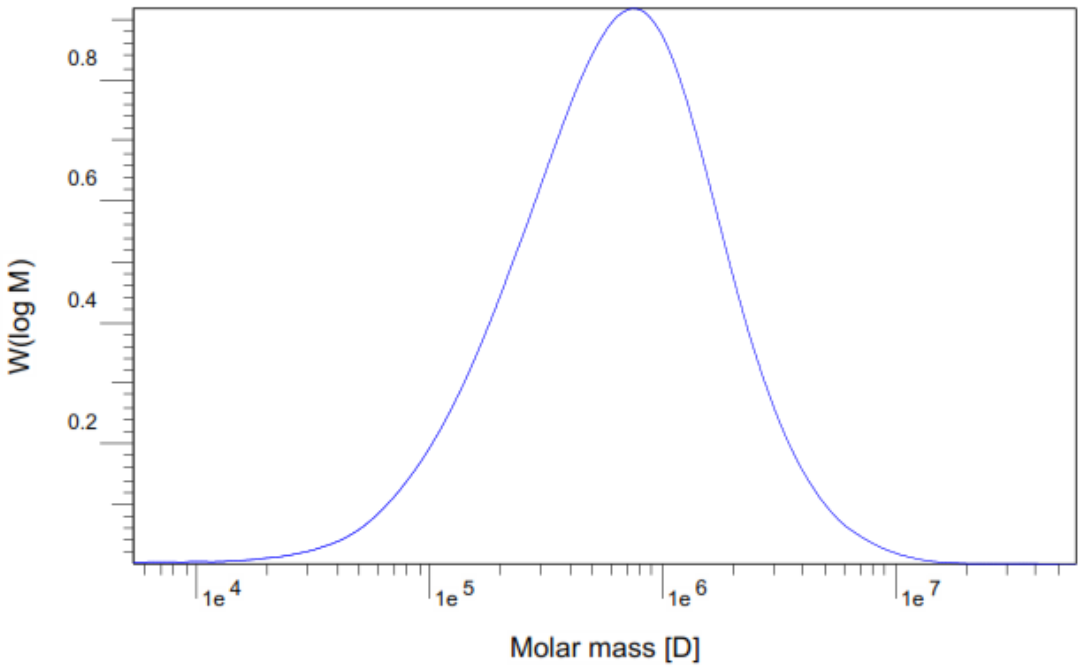


Fig. 3. Gel permeation chromatography (GPC) for the extracted cyanobacterial PHB.

Depending on various factors such as the production strain, the nutrient supply, and the cultivation parameters biosynthesized PHA polymer chains may have 10^2 to 10^5 3HA monomers. PHB consists merely of 3HB subunits, featuring rather high crystallinity and restricted processability. The low difference between the decomposition temperature and the high melting point provides a window of processability which is too narrow for many processing techniques (Koller et al., 2013). These obstacles can be overcome by interrupting the crystalline PHB matrix by additional building blocks like 3HV and 4HB, resulting in improved material properties and a broader range of applications (Koller et al., 2013).

Unpublished data

Besides the raw material used and the process specifications, downstream processing for polymer extraction and recovery is a rather cost-determining factor in the PHA production process. Research needs more focus on the downstream processing of PHA making the process more sustainable and economically feasible.

PHA, poly(lactic acid) (PLA), polyethylene (PE), poly(trimethylene terephthalate) (PTT) and poly(*p*-phenylene) (PPP) are the best-studied polymers containing at least one monomer synthesized via bacterial fermentation (Chen, 2010). Among which PHA and PLA are substantial for their biodegradability and PHA is the only polymer which is completely synthesized in vivo. **Table 1**represents various bacterially synthesized polymers and their thermal properties and molecular weights. PHA has the most versatile structure, resulting in most variable Mw, T_m,T_g, and thermo-degradation temperature (Chen, 2010; Wang et al., 2009). Incomparison, the low-cost PLA is brittle with an elongation at break of 5.2–2.4%, yet its tensile strength is the highest among these bacterial plastics, ranging from 49.6 to 61.6MPa, and $T_g$of 60°C has been a weak point for PLA application as articles made from PLA change shape at this temperature(Chen, 2010; Żenkiewicz et al., 2009).

The polymer obtained in this study represents an adequately high molecular weight and polydispersity index. In order to offset the relatively high production cost of the polymer and to further improve the physical properties of the material can be blended with other polymers. Blends of PHA with natural fibers were found to have improved mechanical properties (Avella et al., 2000)

Unpublished data

Table 1. Comparison of various bacterial polymers in thermal and molecular properties.

Polymer composition	Thermal Properties			Molecular Properties			References
	[a] T_m °C	[b] T_g °C	[c] T_c °C	[d] Mw $gmol^{-1}$	[e] Mn $gmol^{-1}$	[f] D -	
PHB by cyanobacteria and CO_2 as substrate	151.42	48.51	73.11	1051900	316060	3.328	(This study)
3HB-3HV copolymer by *Cupriavidusnecator* from mix of plant oil	168	-0.9	N.D	N.D	670000	3.9	(Lee et al., 2008)
3HB-3HV by mutant *Cupriavidusnecator*	89	-16.1	N.D	N.D	N.D	N.D	(Bhubalan et al., 2010)
PLA (chemically synthesized)	175	60	N.G	5-50 $*10^4$	N.G	1.8-2.6	(Chen, 2010)
PE (Chemically synthesized)	136.4	33.43	N.G	10-600 $*10^4$	N.G	2.1-6.8	(Chen, 2010)
PTT(Chemically synthesized)	227.55	42.6	N.G	3.8 * 10^4	N.G	2	(Chiu & Ting, 2007)
PPP (Chemically synthesized)	N.D	173-232	N.G	0.4-33 * 10^4	N.G	1.5-3.6	(Chiu & Ting, 2007)

[a]Melting temperature

[b]Glass transition temperature

[c]Crystallization temperature

[d]Molecular weight

[e]Number average molecular weight

[f]Polydispersity index

N.D = not determined & N.G = not given

PLA = Poly(lactic acid)

PE = Polyethylene,

PTT = Poly(trimethylene terephthalate)

PPP = Poly(*p*-phenylene)

Today, the market share of bioplastics is approx. 1% and price-driven mass markets such as packaging are difficult to enter (compare film for food packaging, which is often a multilayer material). Applications for PHA range from new fields such as additive manufacturing (filament) to high end (and less price sensitive) therapeutic use. PHA show both advantages of biobased carbon content and full biodegradability, also in the marine environment, and compared to PHA from carbohydrate fermentation, cyanobacterial PHA can be more

sustainable and more cost effective. It can be carbon-negative material, making the process not only attractive for PHA converters and users, but also for CO_2 emitters like power stations. There are plenty of medium-size CO_2 point sources, e.g. biogas production facilities, where the CO_2 could be used in an adjacent cyanobacterial PHA factory erected on non-arable land. Best would be a biorefinery approach, where valuable compounds such as phytohormones and pigments are extracted from the cyanobacteria, then PHA and finally the biomass is anaerobically digested in a biogas plant, yielding a cost-effective, fully integrated process.

4. Conclusions

PHA production from CO_2 has the potential of reducing the production cost of this biodegradable polymer and also making the material more sustainable and competitive in the market with other bioplastics. Here, the PHB produced from CO_2 using cyanobacteria as cell factory was characterized and the potential of the polymer for the commercial production was established. The chemical modification of bacterial PHAs is a key method for the commercialization of valuable polymers for specific applications.

Declaration of the author's contributions

DK and MK have performed the extraction and purification of the polymer from cyanobacteria and DK has carried out the quantification of PHB and written 60 % of this manuscript. RI has performed the DSC, FTIR and GPC analysis with the supervision of CP and has prepared the report for the analysis. IC has performed the DMTA analysis (results not included yet). The study has been initiated by ML and CH and CH has supervised this project.

Conflict of interest

The authors have no conflict of interest to disclose.

Unpublished data

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable

Funding

“This research was funded by the authors’ institutions”.

Unpublished data

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6) Conclusions

Photosynthetically produced PHB is the hotspot for researchers as the most viable potential alternative to the non-biodegradable polymers. Due to the economic disadvantages of cyanobacterial PHB their usage in the market is abandoned. Hence, the development of an economically superior bioprocess for the production of cyanobacterial PHB is of the foremost importance. For which a systematic and quantitative approach to facilitate understanding of the process is needed. The starting point is the choice of a robust, high polymer producing and industrially optimized strain which is crucial for a successful production process. Many unexplored cyanobacterial wild-types and wastewater born strains are yet to be explored for their potential for photosynthetic PHB production. Once a strain or a consortium of photosynthetic microalgae has been screened and selected for PHB production various methods can be applied for further improvement of the production. Random mutagenesis can be applied as an alternative method for genetic engineering to generate more efficient, robust and economically feasible strains with superior characteristics. Random mutagenesis has the potential of producing strains with characteristics such as tolerance to high salt concentrations and fluctuation in temperature, light intensity and pH changes, strains which will be suitable for outdoor unsterile cultivation conditions. The physiological responses to media availability and therefore media optimization has to be done to maximize productivity and minimize the cost of media. Finally, to check the actual potential of photosynthetically produced PHB, the cultivation of cyanobacteria has to be done in pilot-scale photobioreactor or an open-pond system using the proper, systematic transfer of knowledge from the laboratory to the industry.

The main achievement of this thesis are as follows:

- A review of the state of the art and challenges associated with cyanobacterial bioprocess engineering.
- Unexplored potential of wild-type cyanobacteria to increase phototrophic PHB productivity, as an example the unicellular strain *Synechocystis* sp. PCC 6714 was presented as photosynthetic PHB producer.
- Quantitative, time-resolved characterization of the process using defined controlled conditions of a photobioreactor helps facilitates process optimization. The approach significantly increased the polymer productivity in *Synechocystis* sp. PCC 6714.

- Random mutagenesis shows huge potential for the development of industrially relevant strains for PHB production and can help identify target genes for future genetic engineering. The selected high PHB yielding mutant, MT_a24 was generated by UV-mutagenesis showing exceptional fitness.
- Understanding the physiological responses to media limitation is essential to optimize and scale production. An easily scalable, one-step process could be established with increased PHB productivity in preference to the commonly done two-step processes.
- A bioprocess with higher productivity and economic feasibility could be developed within this thesis.
- Quality assessment of the PHB extracted from cyanobacterial show potential of photosynthetically produced polymer for the production of biodegradable plastics in extended applications.

6.1 Cyanobacteria as production platform-characteristics of an industrial strain and major challenges in strain improvement

One of the main challenges for cyanobacterial products to enter the market is that cyanobacterial strains are not yet optimized as cell factories for industrial processes. While intensive research has been done over the past 20 years for cyanobacterial strain improvement, they have not found their way into the market yet. To realize an economically feasible cyanobacterial PHB production platform, the following strain improvement strategies are essential: development of a state of the art tool for genetic modification, creating more robust industrial strains tolerant to harsh cultivation conditions, increasing the photosynthetic efficiency and the productivity and reduction of overall process and cultivation costs.

In case direct genetic engineering strategies cannot be applied, generation of mutant strains using random mutagenesis can be done. In this case, a mutagen agent such as UV-light or chemicals can be used causing transversions in the genome (Jaeger, 2015). The main challenge, in this case, is the comprehensive screening for the desired phenotypes.
The other substitute technique to generate mutants is insertional mutagenesis, in which a selection marker is randomly introduced in the host genome DNA. When this selection marker is introduced in a gene, the gene will code for a non-functional product (Jaeger, 2015) and the exact mutation can be identified using PCR, as the sequence of the selection marker is previously known. The main disadvantage of insertional mutagenesis is the mutants are considered as genetically modified organisms.

The strain MT_a24, a randomly mutated strain of *Synechocystis* sp. PCC 6714 has great potential as PHB producer. It has shown high PHB productivity under the limitation of

nitrogen and phosphorus (Kamravamanesh et al., 2018). Even though the selection for the best strain from the library of randomly mutated strains was a laborious task, the outcome was a strain with a pool of outstanding characteristics. The PHB productivity was increased in MT_a24 by 2.5-folds obtaining values of $37 \pm 4\%$ (DCW) polymer and maximum volumetric PHB productivity of 134.2 mg L^{-1} d^{-1}. Besides the production of PHB, the MT_a24 produces significant amounts of glycogen under optimized cultivation conditions (Kamravamanesh et al., 2019).

Some tools will be developed in the near future to enable engineering of *Synechocystis* for the production of commodity products. These tools will also enable improvement in physiological traits such as temperature and salt tolerance, and photosynthetic efficiency. The CRISPRi based gene editing to overexpress PHB biosynthetic genes has not been yet reported. While the CRISPR-based editing allows the creation of marker-less knockouts and knock-ins. Thus, in the future, the cyanobacterial strains produced might be considered commercially sustainable and safe for outdoor cultivations and CO_2 sequestration.

6.2 Quantitative approach to bioprocess and physiological understanding

Like all other bioprocesses, PHB production from cyanobacteria is mainly influenced by the cultivation parameters and nutrient supply. The importance of defined cultivation conditions used to obtain highly productive process for cyanobacteria and microalgae has been illustrated in this thesis. PHB production in cyanobacteria is mainly induced by nutrient limitation, mainly of nitrogen and phosphorus. Therefore, media optimization plays an important role to maximize the PHB productivity and lower production costs. The optimization of media components, nitrogen, and phosphorus in the case of *Synechocystis* sp. PCC 6714 increased volumetric as well as specific production rates, both in the case of biomass growth and PHB content. The supply of nutrients like nitrogen and phosphorus in limiting concentration leads to loss of photosynthetic efficiency in microalgae. The loss in photosynthetic efficiency under nutrient limitation will reduce the amount of carbon and energy that can be accumulated as PHB. Preventing the loss in photosynthetic efficiency may result in higher PHB productivity since there will be more energy available in the form of ATP and NADPH to be stored as PHB. This can be achieved either by the smart genetic modification tools for microalgae or screening for strains which require no media limitation for PHB accumulation. Then the PHB production process can be done in a continuous mode of operation instead of the commonly done batch or sequence batch reactors.

Besides media limitations, other key parameters influencing PHB production and polymer degradation in cyanobacteria are cultivation conditions, such as temperature, pH, light intensity, or light/dark cycles. The accumulated intracellular polymer is easily degraded to produce ATP and NADPH when the fluctuation in cultivation parameters occur or during the dark cycle.

Random mutagenesis can be applied to generate such insensitive strains which are tolerant to fluctuations in environmental conditions. However, the selection criteria for screening a strain or a cell troop with all aforementioned characteristics remains as a challenge.

6.3 Photosynthetic PHB production in industrial scale

Two primary challenges of entering cyanobacterial PHB into the market are the concern of the sustainability of the production process and the high production costs mainly of fresh water and nutrients. One solution could be to use waste streams like agro-industrial effluents with high nitrogen and phosphorus contents. Therefore, the production of the polymer is accompanied by the removal of nutrients from the water. On the other hand, the undefined substrate may raise new challenges that then need to be resolved

6.3.1 Wastewater treatment practices coupled with cyanobacterial PHB production

Microalgae especially cyanobacteria due to its diversity play an important role in the treatment of water for the utilization of nitrogen sources such as nitrate, nitrite, and ammonia as well as phosphorus and sulfur sources. Even though cultivation in an open system has a number of drawbacks: risk of contamination, high evaporation, and inadequate process monitoring and control, they are preferred for mass production of microalgae. It has been suggested that the use of microalgae in wastewater treatment plants considerably reduces the cost of microalgae cultivation and consequently controls the pollution, conserves freshwater resources and produces useful chemicals and biomass (Lavanya et al., 2017). Besides cost-effectiveness, the advantages of using cyanobacterial PHB production coupled with the wastewater treatment facilities are low energy requirement and greenhouse gas emission reduction. Using flue gasses can reduce the production cost of microalga biomass to around $2.5 \in kg^{-1}$ while using wastewater can decrease the costs further to $2 \in k^{-1}$ (Acién et al., 2012). However, as flue gases may contain growth inhibitory substances, pretreatment is required which may increase the total costs.

Although several photobioreactors have been proposed for the mass production of algae, only a few are practically effective and economically feasible. It has been shown that the total cost of biomass production in outdoor cultivations can be up to 7 times lower than the indoor processes (Wang et al., 2014). Even though effective wastewater resource recovery with microalgae can be challenging due to the variability in the nutrient composition of influent water (Wagner et al., 2017) yet the only way to commercialization of cyanobacterial derived PHB seems to be the use of agro-industrial waste streams and open raceway systems as photobioreactor.

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Part III

Summary

7) Summary

7.1 Published and submitted papers and patents

1] **Kamravamanesh, D**., Lackner, M., Herwig, C., 2018. Bioprocess Engineering Aspects of Sustainable Polyhydroxyalkanoate Production in Cyanobacteria. Bioengineering. 5(4), 111.

2] **Kamravamanesh, D**., Pflügl, S., Nischkauer, W., Limbeck, A., Lackner, M., Herwig, C. 2017. Photosynthetic poly-β -hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714. AMB Express, 7(1), 143.

3] **Kamravamanesh, D**., Kovacs, T., Pflügl, S., Druzhinina, I., Kroll, P., Lackner, M., Herwig, C. 2018. Increased poly-β -hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: Mutant generation and characterization. Bioresour. Technol., 266, 34-44.

4] **Kamravamanesh, D.**, Slouka, C., Limbeck, A., Lackner, M., Herwig, C., 2018. Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: bioprocess understanding and evaluation of productivities. Bioresour. Technol., 273, 277-287

5] **Kamravamanesh, D**., Kiesenhofer D., Fluch S., Lackner M., Herwig C., 2019. Scale-up challenges and requirement of technology-transfer for cyanobacterial poly (3-hydroxybutyrate) production in industrial scale. Under revision at the Journal of Bioresource Technology.

6] Hofer, A., **Kamravamanesh, D.**, Bona-Lovasz, J., Limbeck, A., Lendl, B., Herwig, C., Fricke, J., 2018. Prediction of filamentous process performance attributes by CSL quality assessment using mid-infrared spectroscopy and chemometrics. Journal of Biotechnol., 265, 93-100

7] **Kamravamanesh, D**., Ruschitzka, P., Herwig, C. Formate catalysis from hypersaline environments by a halotolerant Halomonas sp. (WO 2018/037081 A1)

8] **Kamravamanesh, D**., Herwig, C., 2016, Biodegradation of aniline from hypersaline environments using halophilic microorganisms (WO 2018/135010 A1)(EP 3375862 A1)

9] **Kamravamanesh, D**., Mahler, N., Herwig, C., 2017, Biodegradation of organic pollutants using a halophilic archaea (WO patent, Ref, COV161158/2017)

10] **Kamravamanesh, D.**, Pflügl, S., Lackner, M., Herwig, C, 2018, increased biopolymer production in cyanobacterial using random mutagenesis (Austrian patent application No.A68/2018)

7.2 International events, oral presentations and posters

11] The international bioprocess design for microalgae 2016, 8-15.07.2016, Wageningen, Netherlands

12] Poster Presentation: **Kamravamanesh, D.**, Pflügl, S., Lackner, M., Herwig. "Photosynthetic poly-β -hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714", The young scientist's cyanobacterial symposium 2016, 5-7.10.2016, Rostock, Germany

13] Poster Presentation: **Kamravamanesh, D**., Pflügl, S., Lackner, M., Herwig. "Photosynthetic poly-β -hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714", The Danube Vltava Sava Polymer Meeting 2017, 5-8.09.2017, Vienna, Austria

14] Poster Presentation: **Kamravamanesh, D**., Pflügl, S., Lackner, M., Herwig. "Sustainable poly-β -hydroxybutyrate production using cyanobacteria" The Algae Europe 2017, 5-7.12.2017, Berlin, Germany

15] Poster Presentation: **Kamravamanesh, D**., Pflügl, S., Lackner, M., Herwig. "Poly-β -hydroxybutyrate production from carbon dioxide in cyanobacteria" The 6th Conference on Carbon Dioxide as Feedstock for Fuels, Chemistry and Polymers 2018, 15-16.03.2018, Cologne, Germany

16] Oral Presentation: **Donya Kamravamanesh** "Bioprocess development for sustainable poly-β -hydroxybutyrate production", 12th European Symposium on Biochemical Engineering Sciences (ESBES) 2018, 9-12.09.2018, Lisbon, Portugal

7.3 The awards

17] Vienna Energy Globe Award 2018, Place 1, Lackner Ventures and Consulting GmbH, Project: PHB- production from CO_2 and sunlight using Cyanobacteria.

18] Mercur Innovationspreis Austria 2018, 1st Prize, Lackner Ventures and Consulting GmbH, Project: PHB- production from CO_2 and sunlight using Cyanobacteria.

8) Appendix

8.1 published Co-author papers and patents

"Prediction of filamentous process performance attributes by CSL quality assessment using mid-infrared spectroscopy and chemometrics"

Challenges: Complex raw materials are multicomponent mixtures with different bioavailability of their ingredients. Within the framework of quality by design, a detailed characterization is necessary in order to generate sound scientific-based relationships between raw material quality and process performance. Model-based methods are adequate tools to facilitate process understanding which further helps decision making on critical material attributes for complex raw materials.

State of the art: Model-based methods are applied in the field of the biopharmaceutical industry. Data-driven models are generally applied for calibration or classification.

Findings: Model-based methods could be successfully applied for knowledge generation with respect to interlink of raw material quality and process performance on the example of CSL.

Impact of the work: A concrete example of the application of data-driven models for raw material assessment is given.

Citation: Hofer, A., **Kamravamanesh, D**., Bona-Lovasz, J., Limbeck, A., Lendl, B., Herwig, C., Fricke, J., 2018. Prediction of filamentous process performance attributes by CSL quality assessment using mid-infrared spectroscopy and chemometrics. Journal of Biotechnol., 265, 93-100

My contribution: I performed the sample preparation and analytical measurement and data analysis.

"Superior Polyhydroxybutyrtae (PHB) production in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714"

Challenges: Cyanobacterial PHB production from an economic point of view has major drawbacks, the native PHB content and biomass productivity are low.

State of the art: So far there has been no general tool to increase PHB productivity in cyanobacteria. Genetic modification also to achieve an essentially increased PHB productivity has rarely been successful in cyanobacteria as there is a lack of knowledge which genes certainly require modification.

Findings: Random mutagenesis can produce strains with superior characteristics, fit for industrial production processes.

Impact of the work: Demonstration of the novelty of using a direct evolution approach to generate industrially relevant cyanobacterial strains.

Citation: Kamravamanesh, D., Pflügl, S., Lackner, M., Herwig, C, 2018, Superior polyhydroxybutyrate (PHB) production in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714 (Austrian patent application No.A68/2018)

My Contribution: I generated the mutant library, performed the analytics and wrote the patent application.

"Formate catalysis from hypersaline environments by a halotolerant Halomonas sp."

Challenges: Industrial saline waste streams are frequently contaminated with organic pollutants such as formate. If these organic contaminants are removed completely then the saline water has the quality suitable to be used for electrolysis.

State of the art: The chemical or the membrane techniques used in industry for the TOC reduction is expensive and at points inefficient.

Findings: A halotolerant strain of Halomonas sp. can remove formate from hypersaline environments having a pure catalytic effect.

Impact of the work: Demonstration of the novelty of using biological treatment for hypersaline industrial effluents.

Citation: Kamravamanesh, D., Ruschitzka, P., Herwig, C. Formate catalysis from hypersaline environments by a halotolerant Halomonas sp. (WO 2018/037081 A1)

My Contribution: I performed the strain screening and did the experiments with Paul Ruschitzka and write the patent application.

"Biodegradation of aniline from hypersaline environments using halophilic microorganisms"

Challenges: Industrial saline waste streams are frequently contaminated with toxic compounds such as aniline. If these organic contaminants are removed completely for the water bodies then the saline has the quality suitable to be used for electrolysis.

State of the art: The chemical or the membrane techniques used in industry for the TOC reduction of wastewaters are expensive and at points inefficient.

Findings: Biodegradation of aniline from hypersaline environments can be done using biological treatment by halotolerant organisms.

Impact of the work: Demonstration of the novelty of using biological treatment to degrade aniline from hypersaline industrial effluents.

Citation: Kamravamanesh, D., Herwig, C., 2016, Biodegradation of aniline from hypersaline environments using halophilic microorganisms (WO 2018/135010 A1) (EP 3375862 A1)

My Contribution: I performed the strain screening and did the experiments and write the patent application.

"Biodegradation of organic pollutants using halophilic archaea"

Challenges: Industrial saline waste streams are frequently contaminated with organic toxic compounds. If these organic contaminants are removed completely for the wastewater then the saline has the quality suitable to be used for membrane electrolysis.

State of the art: The chemical or the membrane techniques used in industry for the TOC reduction of wastewaters are expensive and at points inefficient.

Findings: The halophilic archaea, Haloferax Mediterranei has the potential of utilizing a wide range of organic and aromatic carbon compounds from hypersaline environments.

Impact of the work: Demonstration of the novelty of using biological treatment to reduce the total organic carbon content of industrial effluents.

Citation: Kamravamanesh, D., Mahler, N., Herwig, C., 2017, Biodegradation of organic pollutants using a halophilic archaea (WO patent, Ref, COV161158/2017)

My Contribution: I performed the strain screening and did the experiments and write the patent application.

8.2 posters

1] Poster Presentation: **Kamravamanesh, D.**, Pflügl, S., Lackner, M., Herwig. "Photosynthetic poly-β -hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714", The young scientist's cyanobacterial symposium 2016, 5-7.10.2016, Rostock, Germany

2] Poster Presentation: **Kamravamanesh, D**., Pflügl, S., Lackner, M., Herwig. "Photosynthetic poly-β -hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714", The Danube Vltava Sava Polymer Meeting 2017, 5-8.09.2017, Vienna, Austria

3] Poster Presentation: **Kamravamanesh, D**., Pflügl, S., Lackner, M., Herwig. "Sustainable poly-β -hydroxybutyrate production using cyanobacteria" The Algae Europe 2017, 5-7.12.2017, Berlin, Germany

4] Poster Presentation: **Kamravamanesh, D**., Pflügl, S., Lackner, M., Herwig. "Poly-β -hydroxybutyrate production from carbon dioxide in cyanobacteria" The 6th Conference on Carbon Dioxide as Feedstock for Fuels, Chemistry and Polymers 2018, 15-16.03.2018, Cologne, Germany

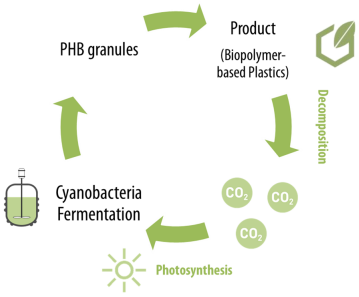
Enhancing PHB Production in *Synechocystis* sp. PCC 6714 by means of Process Engineering and Multivariate Design of Experiments

Donya Kamravamanesh[1,2] | Stefan Pflügl[1] | Christoph Herwig[1] | Maximilian Lackner[2]

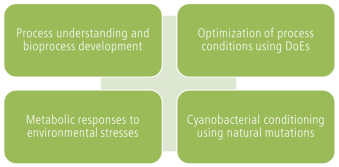
[1]*Vienna University of Technology, Institute of Chemical Engineering, Research Division Biochemical Engineering, Vienna, Austria.*
christoph.herwig@tuwien.ac.at
[2]*Lackner Ventures & Consulting GmbH, Barbenweg 1A, 1220 Vienna, Austria*

BACKGROUND

High cost of raw materials for the processing makes PHB expensive in comparison to petroleum-derived plastics. Cyanobacteria are the promising platform for production of PHAs under nutrient (P, N) limitation from sustainable resources sunlight and CO_2. Major limitation in large scale cultivation is the slow growth. Although optimization of cultivation conditions in photobioreactors could resolve this bottleneck.



MOTIVATION AND APPROACH



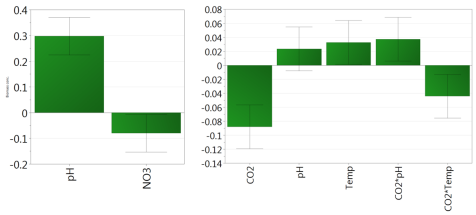
- The goal of the study is to enhance PHB production in *Synechocystis* sp. 6714 using multivariate experimental design and process engineering.
- Development of an economically superior process with optimized conditions to enhance biomass growth and PHB productivity in cyanobacteria.
- Reveal *Synechocystis*/physiology under nutrient "replete" vs " deplete" conditions.

METHODOLOGY AND FINDINGS

DoEs in shake flasks (Influential factors for *Synechocystis* cultivation)

CO_2 as the sole carbon source for biomass growth and PHB productivity

Effect of process parameters on biomass growth and PHB content			
Factor	Low	High	Middle
pH	6	10	8
Nitrogen conc. g/l	0,15	1,5	0,825
CO_2 conc. %	5	25	15
Temperature	25	35	30

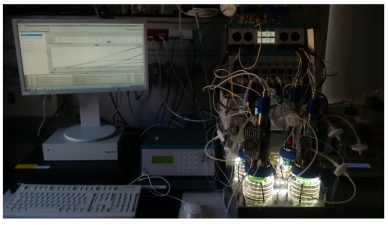


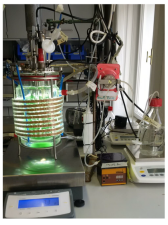
Results

- Significance of pH and nitrate concentration on biomass growth. Higher biomass concentration at higher pH.
- Higher CO2 concentrations leads to lower final biomass concentrations and PHB content.
- Quadratic effects of CO2*pH and CO2*temp on biomass concentration and final PHB content.

Ongoing studies for physiological characterization of *Synechocystis*

- Light regulated bioprocess development and optimization of process parameters
- Rate limiting factors for biomass cultivation
- Targeted nutrient limitations under optimized conditions
- Bioprocess scale up in a photobioreactor, PHB recovery and improvement of product titer





CONCLUDING REMARKS

Significance of process parameters on biomass growth and PHB accumulation in *Synechocystis* sp. 6714.
Feasibility to use CO_2 for cyanobacterial biomass production under optimized process conditions.
Optimization of cultivation conditions in photobiorecators can pave the way towards feasible industrial scale PHB production.

Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714

Donya Kamravamanesh[1,2] | Stefan Pflügl[1] | Maximilian Lackner[2,3] | Christoph Herwig[1]

[1]Vienna University of Technology, Institute of Chemical, Environmental and biological Engineering, Research Division Biochemical Engineering, Vienna, Austria.
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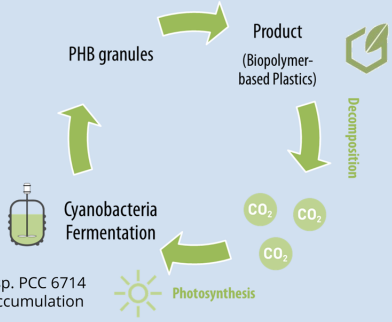
[2]Lackner Ventures & Consulting GmbH, Barbenweg 1A, 1220 Vienna, Austria
[3]University of Applied Sciences FH Technikum Wien, Vienna, Austria.

BACKGROUND

Poly-β-hydroxybutyrate (PHB) production from CO_2 has the potential to reduce the production cost of this biodegradable polyesters, and also to make the material more sustainable compared to utilization of sugar feedstocks. Cyanobacteria are the promising platform for production of PHAs under nutrient (P & N) limitation from sustainable resources sunlight and CO_2. The major limitation for the large scale cultivation is the slow growth and insufficient productivity.



MOTIVATION AND APPROACH

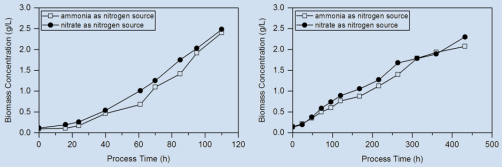
- Process understanding and bioprocess development
- Optimization of process conditions using DoEs
- Metabolic responses to environmental stresses
- Cyanobacterial conditioning using natural mutations

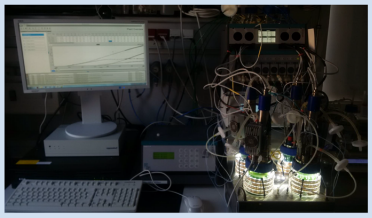
- The goal is to study the unexplored unicellular cyanobacterium *Synechocystis* sp. PCC 6714 for biomass growth and PHB accumulation under nutrient limitations.
- To enhance growth rate and PHB productivity in *Synechocystis* sp. 6714 under controlled defined conditions of a photobioreactor.
- Development of an economically superior process with optimized conditions to boost biomass growth and PHB productivity in cyanobacteria.

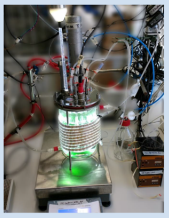
METHODOLOGY AND FINDINGS

Shake flasks vs. Photobioreactor cultivations of *Synechocystis* sp. PCC 6714

Biomass growth on two nitrogen sources nitrate and ammonia and CO_2 as the sole carbon source



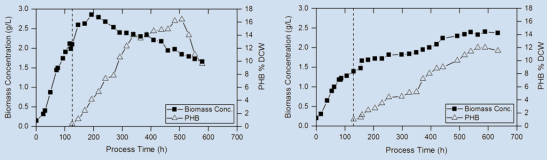




- Growth rates and biomass concentration are 5 folds higher under defined conditions of a photobioreactor

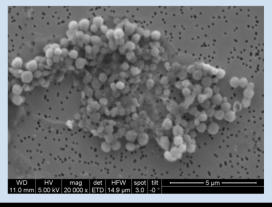
PHB accumulation under controlled defined conditions of a photobioreactor

- Two step cultivation of *Synechocystis* sp. PCC 6714 under nitrogen and phosphorus limitation
- Growth on two different nitrogen sources
- Targeted nutrient limitations under optimized conditions



Results

- Under controlled defined conditions of a photobioreactor *Synechocystis* sp. PCC 6714 accumulates up to 18% PHB of dry cell weight
- Highest volumetric production rate of PHB was 59 ± 6 mg $L^{-1}d^{-1}$
- Using bioprocess optimization tools we have been successfully able to enhance the PHB productivity to maximum of 160 mg $L^{-1}d^{-1}$ from CO_2
- Scanning electron microscopy of *Synechocystis* sp. PCC 6714 showed electron transparent PHB granules of nitrogen and phosphorus limited cells with an average diameter of 0.7 µm



OUTLOOK

- For the process to make sensibility from economical as well as ecological point of view further increase in productivity seems necessary
- Our focus is strain improvement using natural mutations, bioprocess optimization tools and media development
- We avoid genetic engineering in order to enable future possibilities of outdoor open-pond cultivations

Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium *Synechocystis* sp. PCC 6714

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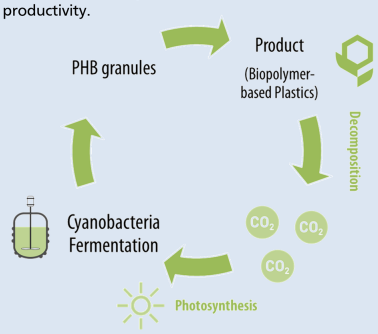

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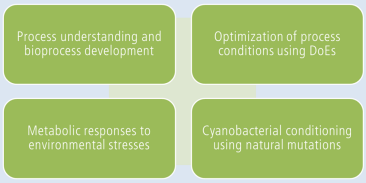

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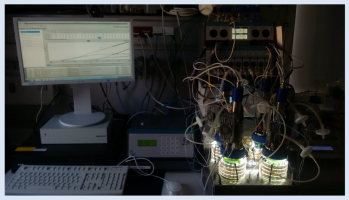

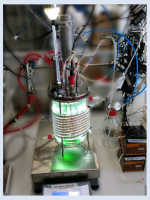

METHODOLOGY

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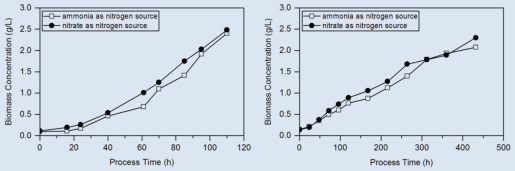

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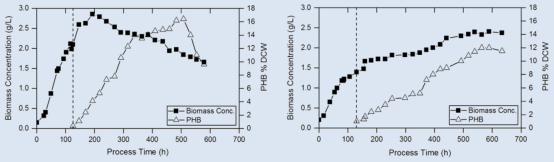

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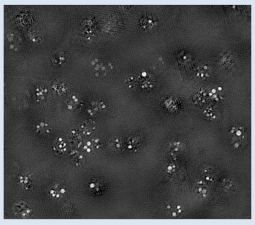

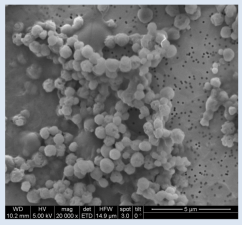

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8.3 Curriculum Vitae

Donya Kamravamanesh

Personal Data

Place and Date of Birth:	Tehran, Iran- 27 July 1985
Address:	Sonnenallee 41/L403, 1220, Vienna, Austria
Phone:	+436606791419
email:	donya_k@outlook.com

Education

December 2014 - present	PhD Student at TU Wien, Austria
October 2008 - July 2010	Master of Biotechnology at University of Pune, India
May 2004 - May 2008	Bachelor of Chemistry at Fergusson College, India

Work Experience

January 2019 - present	Project Assistant at TU Wien, Austria
Sep 2015 - Dec 2018	Project assistant at Lackner Ventures and Consulting, Austria
Dec 2014 – Aug 2015	Intern and PhD student at TU Wien, Austria
Sep 2012 – Dec 2014	Tutor at TalayehDaran School, Tehran, Iran
Aug 2010- June 2012	Internship and project assistant at International Cell Center, India

Skills

IT	MS-Word, MS-Excel, MS-Powerpoint, MS-Project, Origin Lab, MODDE, Lucullus
ANALYTICS	HPLC, CHROMELEON, Spectroscopy, Microscopy
LANGUAGES	English, Persian, Hindi, German (Beginner)